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UNSOLVED PROBLEMS OF GENERAL BIOLOGY[1]

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In 1895 Delage[2] wrote as follows:

La Biologie générale comprend, malgré son nom, un grand nombre de problèmes particuliers. La division cellulaire, le mouvement du protoplasma, la formation des espèces, ne sont pas certes des questions mesquines; cependant on peut entreprendre de les résoudre sans connaître la constitution intime de la substance organisée et ses modes d'action. Ce sont, malgré leur ampleur, des questions particulières. La Biologie renferme quelque chose de plus général, c'est ce qui concerne l'essence même de la vie, comment elle commence, se continue et se transmet.

Il n'y a pas deux manières d'attaquer le problème de la vie pour en trouver une solution positive. La vie résulte évidemment des propriétés de la matière vivante et ces propriétés sont le résultat de sa constitution chimique. Or là, nous sommes radicalement arrêtés. La chimie nous enseigne la composition élémentaire du protoplasma; elle nous fait connaître un certain nombre des substances complexes qui entrent dans la constitution de ses différentes parties; l'histologie nous montre en lui des organes minuscules fort complexes, associés pour former la cellule; mais entre les molécules, que le chimiste ne fait que peser ou compter, et ces organes déjà si compliqués, il y a une lacune immense. Quel arrangement prennent les molécules? Quelles associations forment-elles pour engendrer la substance vivante?

Notre ignorance est absolue sur ce point.

There is no doubt that, at the time of this writing, this statement correctly expressed the profundity of our ignorance concerning the chemical structure of living protoplasm. The recognition that certain elements, extracted from living substance—such as carbon,

[1] This is the second of an invited series of synthesizing papers. Others, which will appear in future numbers, include the following:

J. H. Bodine, Exact title to be announced later—some phase of cellular physiology.

Alan Boyden, "Serology and Animal Relationship."

L. V. Heilbrunn, "The Relation of Protoplasm to the Calcium Ion."

R. S. Lillie, "Artificial Parthenogenesis in Relation to the Problems concerning the Initiation of Development."

B. H. Willier, "Embryonic Organization in Presomite Chick Blastoderms."

[2] Y. Delage, *La Structure du protoplasma et les théories sur l'hérédité et les grands problèmes de la biologie générale* (Paris: Reinwald, 1895).

hydrogen, oxygen, and nitrogen—enter into the structure of carbohydrates, lipins, and proteins did not appreciably dissipate this ignorance. Nor could it have done so, since the living substance is a complex comprising not only these organic compounds but also water and electrolytes. The structure and behavior of living substance are derived from the reactions embracing elements acting singly or with each other and with the organic compounds and water. Upon these combinations no resolution into elements can throw any light, since an analysis of a mineral water into constituent elements does not tell us what compounds of them are present. Protoplasm, far more complex than a mineral water, resolved into constituent elements, or into compounds even, is no longer protoplasm.

After forty-five years this gap in our knowledge persists. Despite the undeniably rich contributions of biochemistry, which have increased our knowledge of the organic compounds, especially that of the proteins which enter into the structure of the cell—and despite the elegant studies of the effects of electrolytes on vital behavior, the vast array of researches on the permeability of protoplasm to water and to solutions, and the formidable attack on other cell problems by biophysicists—we are far from understanding, on the basis of protoplasmic structure broken down into elements, protoplasm as a living self-perpetuating system. Between the supposed beginning, the elements, and the end-point, the cell, we have, in default of exact knowledge, only vague conceptions. No less has orthodox biology, which deals with the morphology of the cell as such, failed to grasp the meaning of this gap in our knowledge.

This ignorance being admitted, the above-quoted statement is of an incontestable fact. It may be otherwise with Delage's judgment that the grand problems of general biology are pendant to the problem of the structure of protoplasm. Some, perhaps many workers, may disagree with it. To me, however, it seems even more apparent today than it was in Delage's time that the only approach to the elucidation of the great riddles of what I denominate "general biology" is by way of knowledge of the structure of protoplasm. The successes—or, more correctly, the "failures"—of theories of general application emanating from particular studies on one cell component, e.g., the chromosome, are unmistakable signs that we need recourse to a practicable principle which envisages life-phenomena in the entire confines where life resides.

The term "general" connotes, for me, the quality of commonness. Thus, "general embryology of animals," for example, accurately speaking, should embrace only those phenomena common to all animal eggs, such as maturation of the egg, its cleavage (early or late), and the delimitation of ectoderm and endoderm—the sole exhibitions commonly displayed by animal eggs whereby they transform themselves into embryonic primordia. Similarly, the term "general physiology" ought to include only phenomena common to both animals and plants. But many a most excellent textbook on this subject discusses nerve and brain, blood, blood vessel and heart, and muscle of all kinds. Since no plant possesses any one of these and many animals lack one or all of them, we may explain this widespread practice as legitimate for teaching purposes. In a more strictly scientific essay one cannot be too exact—least of all in a science in which definition means so much can one use terms loosely. In this wise I consider the term "general biology" to include all those phenomena revealed to us by both animals and plants, and only those phenomena. How living things come to be and how they maintain themselves and transmit themselves constitute the chief problems common to all animals and plants; they are problems of general biology.

Now the visible or tangible expressions of livingness, i.e., the phenomena of the life-state, do not, so far as we know, exist apart from that material configuration which we denominate "protoplasm." Therefore, when one agrees that "matter as such produces nothing, changes nothing, does nothing"[3]—although the "as such" saves us, if it does not beg the question—one does not escape the necessity of seeking to know what the substance called "protoplasm" is, and how it acts and is acted upon as center and object of force and as seat of energy. The very fact, as stated, that "when we deal with matter in the concrete force does not, strictly speaking, enter into the question, for force, unlike matter, has no independent objective existence"[4] demands of us a knowledge of the concrete substance "protoplasm." While it would be a mistake to exaggerate this concreteness, especially by too great an emphasis on static qualities, it would be an even greater error to supplant the concrete system by a subjective and symbolic term. On what, in what, and with what, force acts is the major problem for him who deals with life as something tangible and objective.

Either we conceive of life in each species of animal and plant as *sui generis*, or we associate the all-pervading life-condition with a common factor. The former point of view renders futile any attempt to reduce life-processes to a common basis on which we may hope to understand those phenomena that experience tells us separate the living from the nonliving. The latter, however, urges us to seek in all life-exhibiting states for common properties, a common substratum in which reside plant and animal characteristics, and, in the members of each of these kingdoms, their specific qualities. It is from this latter point of view that I envisage the problems of general biology. Living things have a common denominator, a basic structure, in which life resides and from which vital manifestations arise. Then life, as force, principle, or what you will, is associated with an organization, a discrete pattern which is common to all living things, be it plant or animal. The one basic feature common to all living things whose phenomena, tangible manifestations, we may apprehend, I conceive resident in protoplasm. As that which sets off the life-state from nonlife, protoplasm is peculiar to living things. We need to know what this substance is without which life does not reveal itself to us, in order to approach an understanding of the grand problems of general biology. These thus center around the structure of protoplasm, conceived not merely as static "matter as such" but as that which by its very impermanence gives life, or, rather, that succession of states which in their ensemble give livingness.

Here I would enter an exception: to amend the foregoing statement of Delage to read "protoplasmic system" instead of "cell." With no wish whatsoever to appear pedantic, I must, nevertheless, in a discussion of the ground-postulate of general biology insist upon clear definition. "Protoplasmic system" and "cell" not being synonymous, I reserve the term "cell" for that protoplasmic system which is composed of cytoplasm and discrete nucleus. This definition recognizes that some protoplasmic systems exist which do not contain a discrete nucleus, although they may possess nuclear matter in a granular or diffuse state. The question of the existence of protoplasmic systems devoid of nuclear stuff remains open. In view of the existence of the various types of protoplasmic systems we may say that a discrete nucleus—or several nuclei, as in syncytia—is not a *sine qua non* of living protoplasm. Strictly speaking, therefore, the structure of

[3] D'Arcy Wentworth Thompson, *On Growth and Form* (Cambridge, 1917), p. 14.

[4] *Ibid.*, p. 11.

nucleated protoplasm does not come within the domain of general biology, as we have defined "general biology" above. This is more than a point for special pleading. Since there are bacteria, algae, and perhaps protozoa, without discrete nuclei, although diffuse or granular nuclear material may be present in them, we cannot dismiss such forms of protoplasmic systems as "exceptions." They are living things and display their life-activities within the framework of protoplasmic structure. In the following pages I keep in mind that biologists recognize the existence of nonnucleated living substance, although I discuss, in the main, the protoplasmic system possessed of a discrete nucleus. This exposition concerning the structure of the protoplasmic system "cell" may serve as an orientation for more profound study of other protoplasmic systems which so far have not received the attention they merit in the interest of furthering knowledge of general biology.

Study of the living cell may be pursued from any one of three points of view: the biological, including the morphology and the physiology of form and form changes; the chemical, involving composition and chemical changes; or the physical. No one of the three is exclusive of the other; each is an auxiliary of the others. But, given the fact that life is ever associated with a kind of organization, is never revealed apart from this organization peculiar only to living things, the first duty of the biologist is the recognition of the concreteness of the life-state, its existence as objective, as an entity. Certainly—in the case of living cells, at least—it is as a tangible mass of substance that they attract attention; we may advance farther in our investigation of these forms of the protoplasmic system than of those that are nonvisible and intangible. Therefore, no matter what the special field of study, the first problem is to know fully the living cell from the biological point of view.

I

As defined above, the cell is that form of protoplasmic system which comprises cytoplasm and a discrete nucleus. Although in this exposition I limit myself to the discussion of animal cells, emphasizing especially egg cells, what I set forth none the less holds true also for plant cells.

Of the living cell, the extra-nuclear component is generally the more important quantitatively. In the longest nerve cells of the human body and in egg cells, the amount of nuclear stuff is less than the cytoplasmic. In spermatozoa the fact that nuclear volume is greater than cytoplasmic perhaps runs with the great degree of specialization that limits so sharply their functions and viability. However, not the whole content of the cell external to the nucleus is living cytoplasm. The first question to be answered, therefore, is: What in the perinuclear area of the cell is living substance par excellence?

The whole of any protoplasmic system, I hold, is a continuous hyaline menstruum, the ground-substance. According to this conception, in the case of cells the ground-substance pervades both nucleus and cytoplasm. It is this menstruum alone that I consider as living substance.

With respect to the cytoplasm, arguments may be adduced from both descriptive and experimental biology to support the proposition that only its ground-substance is living.

In tissue cells, such as secreting cells, visible granules appear and disappear according to states of cellular activity. Their disappearance may also result from experimentally induced stimulation. In fatigued cells or in cells suffering from lack of food, granules are less numerous. Since fat is transported across the intestinal wall, oil globules appear

most abundantly in the cells of the intestine. Thus, certain types of formed bodies in cells arise as products of metabolism or are food materials. Others are undoubtedly merely effete material. No one would denominate, as "living," particles of carmine, of ink, or of iron filings introduced into a *Paramecium*. So, other inert materials entering or leaving a cell, as well as those due to secretion and excretion, are not necessarily living because they are discerned within the cell boundary.

An animal egg cell at the normal moment of its fertilization is richly freighted with various cytoplasmic inclusions, such as oil, yolk, mitochondria, pigment granules, etc. But it did not possess all these at the time when it began its history as a primordial germ cell. Indeed, at the outset it is often difficult to distinguish, among primordial germ cells, which is to be egg cell and which sperm cell. True, many germ cells show differentiated cytoplasm, as may be determined early in the cleavage cycle by virtue of some form of granules in the cytoplasm, as in the blastomeres of the egg of *Sagitta*, *Cyclops*, etc. On the other hand, eggs of *Ascaris* show no such cytoplasmic indicators. There is, therefore, no ground for supposing that the presence of a cytoplasmic inclusion determines that a cell will become germ cell; rather, the germ-cell indicator, if such is present, may be an expression of the ground-substance itself.

Among the trematodes and cestodes, the yolk is elaborated by accessory vitelline glands and added to the egg. Here vitelline material and egg, as products of the ovary, are sharply separated. It is legitimate to consider this as no special case; rather, it is comparable to the mode by which the yolk is deposited in the egg of *Dytiscus*, of *Diopatra*, of mammals, etc. That is, during its cycle from primordial germ cell to definite egg cell, the egg elaborates reserve from food outside itself. Its specific quality of cytoplasm determines the disposition of the food so gained and the conversion of this into inclusions which themselves show some degree of specificity.

After the initiation of development, fertilization, or parthenogenesis the egg sunders itself into blastomeres. Some of these have more inclusions than others; among these latter are those whose cytoplasm appears in life as optically empty. But all—the richly laden and the optically empty—are living cells. Indeed, the tempo of life-processes is faster in those cells whose cytoplasm is less laden.

Many animal eggs exhibit in striking degree a characteristic pattern in a measure determined by the differential location of the formed bodies in the cytoplasm. These bodies, of different chemical constitution and physical attributes, occupy normally in the unfertilized egg positions independent of their specific gravity. In some eggs they are, after initiation of development, shifted to new cytoplasmic areas; this relocation cannot be ascribed to a sorting-out of bodies according to their specific gravity, for the lightest components, the oil drops, do not come to lie separately from the heaviest, the pigment, situated below the yolk spheres. Rather, the distribution, ever the same—especially a facile demonstration in eggs, as of *Chaetopterus*, or of the nereids, whose cytoplasm includes formed bodies of great variety as to form, size, and color—is without reference to the specific gravity of the inclusions. Oil and yolk are juxtaposed in these eggs with determinate cleavage; or, as in the egg of *Arbacia*, the oil drops are shuttled back and forth, now closely agglomerated, now more widely dispersed, during the cycle of mitosis, while the yolk is packed in the vegetal hemisphere of the egg, and the pigment granules are trapped at the egg surface. In other eggs, such as those of elasmobranchs, reptiles, and birds, to a disk of cytoplasm, which in the living state appears devoid of formed bodies, is pendent the mass of yolk and oil. In eggs, of cephalopods and of

teleosts a thin layer of superficial clear cytoplasm becomes, after fertilization, converted into a similar disk at the surface of the yolk mass; whereas in eggs of insects, a clear rim of surface-located cytoplasm incloses a mass of yolk and other formed bodies.

In eggs of some species of animals the granules suspended in the cytoplasmic menstruum appear to occupy positions in the unfertilized egg, when undisturbed, which are constant with respect to a line drawn from the site of polar-body extrusion to the opposite pole of the egg. Eggs exhibiting such distribution of the cytoplasmic inclusions are said to reveal polar organization, the location of these inclusions being an index of the eggs' polarity. The most striking examples of this type of organization, best seen after fertilization, are offered by eggs of the cephalopods, elasmobranchs, teleosts, reptiles, and birds; in these the cytoplasmic inclusions, such as oil and yolk, are pendent to a disk of clear cytoplasm situated below the point of polar-body extrusion, in which disk alone the initial cleavages of the egg occur. In such telolecithal eggs, so-called, one might say, if they are radially symmetrical, that the spherules and granules in the cytoplasm have a disposition indicating an organization of the egg with reference to a line drawn from site of polar-body formation to the egg's opposite pole. This, however, does not mean that in such eggs the distribution of cytoplasmic inclusions determines polarity or that polarity depends upon this distribution. The cause of polarity in these eggs is independent of the presence of these bodies. This statement is supported when we consider all animal eggs.

Properly to envisage the problems of general embryology, problems common to all animal eggs, one ought to include every egg known to us by descriptive studies and ought to appraise experimental studies on eggs of individual species with special reference to these general problems. Such study demands a most exact knowledge of normal processes and of what is peculiar and specific to a given egg. We should guard against the mistake of attempting to interpret the grand problems of embryology by descriptive, and especially experimental, study on a single species of egg—viewing, as it were, the whole field of animal embryology through the body of a single egg and obtaining thereby a distorted image of the truth.

Many eggs, both before fertilization and during the cleavage stages subsequent to fertilization or the parthenogenetic initiation of development, never exhibit any constant relation of the arrangement of oil, yolk, and other bodies to an imaginary straight line running from the point of polar-body extrusion to the pole opposite. In such eggs, other indexes are used to discern polarity. The term "homolecithal," applied to eggs in which the yolk spheres are uniformly dispersed, indicates that animal eggs exist in which the distribution of the yolk is not oriented with respect to a polar axis.

One denominates as "centrolecithal eggs" those in which the yolk appears massed around the egg center, as in a medusa or an insect egg. Such an egg, even if it is radially symmetrical, owes its polarity to some other factor than that of the distribution of yolk. The location of the yolk, taken alone, is no evidence for polarity in centrolecithal eggs.

Among flatworms are found some species of eggs to which yolk is added by vitelline glands. Here, polarity presumably is inherent in the organization of the egg. It is unlikely that the adventitious disposition of material from accessory glands determines polarity.

Further, any egg properly denominated as one having a polar axis must obviously exhibit polar symmetry. However, there exist eggs which are bilaterally symmetrical;

these cannot, just as obviously, possess polarity in the sense here discussed—i.e., in them no line can be drawn to the points on which the cytoplasmic inclusions can be related radially. Bilaterality relates to a plane and not to a line; eggs are, moreover, figures in three planes of space, they are never lines or planes.

The distribution of yolk would indicate polarity only in a radially symmetrical telolecithal egg. Since no such egg exists, the location of yolk is neither a factor nor an indicator of polarity in animal eggs. It thus follows that yolk plays only a secondary role in the organization of the egg and in embryogenesis.

This conclusion is further supported when we consider the fate of the yolk sac in the mammalian egg. A vestigial structure, it plays no role comparable to that of the yolk in a cephalopod or in an insect egg or to that of the yolk mass added to a trematode or cestode egg. In lieu of the transformation of yolk into protoplasm, the mammalian egg elaborates protoplasm from the maternal blood.

The situation is therefore this: All animal eggs obtain nutritive substances from without. In some the material metabolized as yolk is accessory to the egg; and in others, as in the mammalian, the preponderance of the source of energy for the stages of development subsequent to the initial period is derived from the blood directly. All this indicates strongly that yolk, as such, is not protoplasm, although it is the raw stuff from which protoplasm builds itself up.

Said otherwise, yolk (and oil) is food material to tide over the developing egg during the period which intervenes after the completion of the energy changes embracing the processes by which the embryo is laid down and before the advent of those depending upon the intake of food. Examples of this type are eggs with total cleavage, such as those of echinids and worms; these utilize no oil and yolk until the gut is laid down. As larval forms, they are unable normally to hydrolyze food given them before the yolk and oil in the gut cells have been broken down. The eggs of teleosts or of birds represent another class, that in which the initial plaque of clear cytoplasm, the seat of the cleavage process, is augmented by the building-up of protoplasm from the subjoined yolk area. The extreme cases, I repeat, are the eggs of some flatworms and the mammalian egg; in the former, the yolk is not a product of the ovary but of an auxiliary gland; in the latter, the egg is denuded of yolk by the formation of the yolk sac. Within these limits one may range all other eggs on the basis of the distribution of the yolk and, better, on the basis of the time during the early stages of development—after complete cleavage, homolecithal eggs; and during cleavage, telolecithal and centrolecithal eggs—when to the clear cytoplasm is added new cytoplasm elaborated from the yolk. The question is not only the spatial distribution of yolk in eggs but also its utilization early or late in the egg's development. From this emanate problems of fundamental significance for the dynamics and energetics of the embryogenetic process. This nonconstant distribution of yolk in animal eggs is a fact to be reckoned with by those who still maintain that cytoplasmic inclusions are, per se, the living units. From this point of view, the yolk of the trematode egg, being extra-ovarian, the egg, as ovarian product, would be auxiliary only; and the yolk sac would endow the mammalian egg with life.

If we envisage more completely the spatial distribution of the yolk in eggs, we note that this is not so varied as the terms "homolecithal," "telolecithal," and "centrolecithal" seem to imply. No animal egg exists in which the yolk is not completely inclosed by the clear layer of superficial cytoplasm, the ectoplasm, itself free of yolk. In all animal eggs the yolk is located in the endoplasm. The centrolecithal eggs encountered

among coelenterates and arthropods are thus no extreme configurations; rather, they represent states in which the conditions common to all eggs are strongly emphasized. That is to say, all animal eggs, except those like the trematode and the cestode egg, are centrolecithal, some more sharply than others, the superficial rim of cytoplasm being more extensive in some than in others. Even the telolecithal egg may be considered centrolecithal, for in it, best seen before the appearance of the cytoplasmic disk, a fine rim of superficial cytoplasm incloses the yolk. Only such eggs as those of the trematode and the cestode are truly alecithal. But their existence tells us that yolk, as such, is not a necessary and absolute essential for the egg cell.

The distribution of yolk thus has no fundamental significance for the egg's development, the types of distribution showing no basic differences. That yolk plays a secondary role in development is also shown by experiment.

Experimentally homolecithal eggs can be rendered telolecithal without any disturbance of the normal course of development. Of the experimental means—staleing, temperature changes, and centrifuging—the last named is best, since by it the egg may be least injured.

If, before fertilization, one centrifuges an echinid egg, such as that of *Arbacia* or that of the polychaete *Chaetopterus*, the cytoplasmic inclusions are massed according to their specific gravity; the oil drops at one pole; the yolk at the other, above pigment granules, the heaviest of the inclusions. Except for the disposition of the oil drops, such a centrifuged egg strongly resembles a telolecithal egg, such as that of a teleost, with the difference, of course, that the centrifuged egg cleaves totally, whereas the teleost egg cleaves only partly. Any egg susceptible to innocuous centrifuging shows the same picture.

Centrifuged eggs whose inclusions show changed locations cleave in all possible manners with respect to the axis of stratification: the first cleavage planes may pass along this axis or at any angle to it. The irrelevance of the plane of division to the axis of stratification is demonstrated also by the position of the polar spindle in eggs fertilizable in the stage of first maturation: in the egg of *Chaetopterus*, for example, the first maturation spindle takes any position with reference to the axis of stratification.

These findings on centrifuged eggs, whether these develop with or without cell lineage, demonstrate sufficiently that the altered disposition of the cytoplasmic inclusions in no wise obstructs the normality of development. The inclusions play a subsidiary role in the embryogenetic process. Indeed, the displacement of yolk, which in the normal egg comes to lie in the gut cells, in no wise interferes with the viability of the larva and indicates that normally yolk-filled cells, now yolk-free, possess the full complement of vital activity. In my preparations of blastulae, gastrulae, and plutei of centrifuged *Arbacia* eggs the yolk is found in mesenchyme, ectoderm, or other cells, including those of the gut. Thus, the yolk is a passive component: displaced to other cells than those of the gut, or lacking to the gut, it does not hinder or vitiate the normal processes of development. The locus of the embryogenetic forces is to be sought in the menstruum in which the inclusions lie. This is the ground-substance.

The occurrence of normal polyembryony, as in insect and armadillo eggs, is evidence of the potency of the clear cytoplasm for the unfolding of the embryogenetic process, for the embryos develop, in the former, from the superficially located clear cytoplasm and, in the latter, from the disk of clear cytoplasm. Not only is the clear cytoplasm sufficient

for the transformation of egg to early embryonic fundaments; it is, in normally polyembryonic insect eggs, capable of giving rise to hundreds of embryos.

Experimentally a normally monoembryonic egg, fragmented before fertilization, may, after fertilization of the fragments, give rise to several embryos. The fact that these egg fragments, when fertilized, develop normally no matter which inclusions they may hold indicates again that it is not the inclusions but the material in which they lie, the clear cytoplasm, which is the seat of the developmental forces. The classical work of Delage and others on merogony admits of this conclusion.

If, against these considerations, one raises the objection that in some eggs, notably those with cell lineage, such as the eggs of *Dentalium* and of *Patella*, blastomeres which were separated after first cleavage develop defectively, thus indicating a differential distribution of embryonic potencies—in some cases revealing a spatial pattern of materials at first cleavage which is similar to what would be the one or the other embryonic region in the intact egg—the very clear answer is that in such eggs the polyembryonic condition is at first cleavage already replaced by the restriction to monoembryony. So far, experiments on fragmenting eggs before fertilization and on fertilizing these fragments admit of no categorical distinction between determinate and indeterminate eggs. Fragments of an unfertilized egg of *Chaetopterus*, cleaving with definite cell lineage, develop when fertilized, as well as those of *Arbacia* or other echinids whose cleavage shows no cell lineage. From the fact, as Teitelbaum has shown, that from an egg of *Chaetopterus*, when experimentally treated during the cycle of first cleavage, twin-embryos are called forth, can be adduced a further argument as to the similarity of indeterminate and determinate eggs. This finding and a similar one on the egg of *Tubifex* constrain us to conclude that the polyembryonic capacity of animal eggs is lost sometime after fertilization; eggs differ in time after fertilization when this loss occurs. Elsewhere[5] I have called attention to the two serious sources of error which place under grave suspicion the validity of Hörstadius' results on the development of isolated blastomeres of echinid eggs. But, even if these results are correct, we still need to bear in mind that, so far as we know, all animal eggs capable of experimental fragmentation are, before fertilization, potentially polyembryonic. This potency resides not in the cytoplasmic inclusions but in the menstruum in which they lie, that is, in the cytoplasmic ground-substance.

Experimental results of another category put this conclusion beyond question. I refer to the developmental capacity of inclusion-free egg fragments obtained by centrifuging unfertilized eggs. Although experiments demonstrating that the potency of such clear fractions of cytoplasm (with or without the egg nucleus) is equal to that of the whole egg cytoplasm are all too few, the evidence obtained from them suffices to warrant a conclusion of wide application.

The original work on the development of fragments of nearly inclusion-free cytoplasm was done by Lillie on the egg of *Chaetopterus*. Later, Wilson confirmed and extended Lillie's observation. These workers found that the unfertilized egg, when centrifuged, is first drawn out into a thread; by further centrifuging, the thread is broken. Thus are obtained from an egg one fragment containing all the inclusions save the oil drops, and one, the clearer fragment, containing the oil drops, which Lillie mistook for the residual substance of the germinal vesicle. Lillie's work clearly showed that the hyaline frag-

[5] E. E. Just, "A Single Theory for the Physiology of Development and Genetics," *Amer. Nat.*, Vol. 70 (1936).

ments of the egg of *Chaetopterus* cleave as do whole eggs, exhibiting the same cell lineage. Thus, for this egg at least, it is true, beyond doubt, that the hyaline substance of the cytoplasm separated from the inclusions, save oil, is capable of embryogenesis. Presumably, pieces below a minimum size would not develop into larval worms. What this limit is ought to be determined, because it would furnish an elegant starting-point for the resolution of several interesting problems in embryogenesis, as well as in the biology of the cell.

My own observations, made on echinid eggs, for the most part at Naples, indicate that the clear cytoplasmic fragments are in no wise inferior to inclusion-rich fragments in developmental capacity. To obtain the fragments, I take two lots of unfertilized eggs, one centrifuged, the other not. A sample of each lot is shaken up with bits of a broken glass cover slip. In the case of the centrifuged eggs, it is a simple matter to remove with a pipette the clear fragments, which, however, often contain the cluster of oil drops. This method, very crude though it is, is preferable to that of obtaining the fragments by centrifuging the eggs in sea water plus sugar solution, because of the toxicity of such sugar–sea-water solutions. Perfectly hyaline fragments obtained from centrifuged eggs develop as well as fragments from uncentrifuged eggs. For the egg of *Chaetopterus* I merely follow Lillie's simple method of centrifuging in sea water at that speed and for that time necessary to fragment the egg. By shaking up pieces of clear fragments with bits of a cover slip, I have obtained clear fragments free of oil drops, which are as capable of development as those containing the oil. I have also studied egg fragments of *Amphioxus* with inconclusive results, owing, doubtless, to the rapidity with which this egg loses normality after having come into sea water.

The conclusion of the foregoing is that the real cytoplasm, the living substance per se, is the hyaline menstruum in which the various formed bodies lie. It, with or without the egg nucleus, develops perfectly if fertilized. Thus we conceive of the egg cell as an organization of this cytoplasm, or ground-substance, plus one gamete nucleus. Up to now there is no single datum worthy of credence which indicates that an animal egg, devoid of one or the other germ nucleus, can develop into a larval form, although we know from the work of Yatsu and of others that enucleated egg cytoplasm can cleave.

The conception of the cytoplasmic ground-substance as the living substance par excellence has far-reaching implications.

The normal egg portrays, in one way or another, a kind of orientation of its structural components, as shown by a definiteness of sequence in time and of order of the developmental process in space which fixes the embryogenesis of each species—so definitely that many speak naïvely of the egg, remembering the course previously followed by its innumerable ancestors. This orientation resides in the ground-substance, which shifts the passive nutrient substances suspended therein to new positions. For some biologists this organization is polar. In biology the term "polarity" ought not be used, as in physics, to mean a directed attraction of visible particles; there is no evidence that the formed bodies in the cytoplasm are polarized—any more than there is evidence that in any single species of egg the embryo forms along a line running from one pole of the egg to that opposite. Such an egg as that of a trematode is, strictly speaking, devoid of yolk particles; an egg fragment free of inclusions develops as the whole egg. From this it follows that the orientation, or organization, of the cytoplasm is independent of yolk and other inclusions.

What shall we say of the fact that fractions of eggs, when fertilized, develop as the

whole egg, thereby revealing that they possess an organization identical to that of the whole egg? Does not this behavior, so closely comparable to that of a magnet when cut up into several pieces, each of which retains magnetic power, suggest polarization in the ground-substance? Perhaps. But until we know beyond question that the fragments present "north" and "south" poles along the original egg axis, we cannot here speak definitely. It is just as likely that in the fragments the "north" and "south" poles represent points on the surface of the intact egg, and that the resemblance of the egg's polarity to a magnet is only superficial and without significance. Observation and experiment could put this question beyond the range of doubt.

Or, take the problem of specificity. Doubtless, cell inclusions, such as oil, yolk, mitochondria, pigment granules, are in large measure specific, differing in different species in form and in size, and in physical and in chemical constitution, as can be shown by direct observation on both living and properly fixed eggs by their physical changes under experimental conditions and by chemical analysis. However, this specificity has its origin in the specificity of the ground-substance. Without tedious repetition of what has gone before, I need merely point out that the varying modes by which yolk is stored in eggs or energy is derived, as in mammalian eggs, from the maternal blood, indicate that eggs, except those of trematodes and cestodes, convert yolk into a structure specific to the egg. In this wise eggs build up yolk; yolk never builds up eggs.

Protoplasmic specificity is also indicated by the nucleus in those systems possessing such. Since, however, not all protoplasmic systems contain nuclei, it would not be safe to assert that specificity is the attribute of the nucleus alone. Rather, on the ground of our knowledge of the existence of nonnucleated protoplasmic systems and on the basis of what we know of the elaboration of nuclear matter in eggs during cleavage, we can, with safety, assert that the hyaline ground-substance of the cytoplasm is the locus where nuclear stuff is elaborated and where nuclear specificity has its source. To this point I return later.

By structure, as by activity under normal and experimental conditions, the ectoplasm of eggs also displays specificity. Since no protoplasmic system, cell or otherwise, exists without ectoplasm, we may assume that the ectoplasm plays an important role in specificity. This, however, derives from the fact that the ectoplasm is peripherically located, differentiated, ground-substance. Nuclear and ectoplasmic specificities such as that of the inclusions are thus derivatives.

When one considers the problem of specificity in all its aspects, one can only conclude that specificity—be it of organisms, both plant and animal; of tissues, meaning how in a named species of organism one tissue differs from another; or that form of specificity which we recognize in immunity reactions—resides finally in the fundamental organization of the individual cells which make up the organism. We recognize these kinds of specificity by relatively gross indexes; even the specificity exemplified by immune reactions, since these show themselves to us by indirect means, we may consider grossly indicated.

By external form, color, size, etc., we distinguish one species of animal from another; or by their behavior—in the case of bacteria, for example—we separate forms morphologically closely alike. But these identification characters in themselves do not fundamentally constitute specificity characters. Human fingerprints, so sharply individual, by no means permit us to say that only in the whorls and ridges of the fingers resides specificity. Here the source of the specificity is the same as that which determines that

an egg or a clear fragment thereof develops most strictly along the way that for countless generations eggs of this species have developed.

Within the field of the specificity of a named species of egg—i.e., within the cytoplasmic ground-substance—arise tissues each of which carries, below the commonly employed diagnostics for their recognition, some organization in which alone its specificity has its source. We can distinguish the human parotid gland from the submaxillary or the sublingual, and any one of these from the pancreas, either in the entirety of the gland or by microscopical examination. But in each is a fundamental ground-plan which is the true and the only source of specificity whence arise the indexes of size, form of cell, distribution and quality of the cytoplasmic inclusions, and the activity of the cellular secretions.

So with immunity reactions. The use of the blood of an individual for the purpose of determining immunity to typhoid, for example, does not at all mean that the blood is the primary source of immunity and susceptibility to the disease. Similarly, phagocytosis, even if it were the sole mechanism of defense against disease-carriers, is not limited to the white blood cells only. Why one individual is more susceptible than another to disease, drugs, etc., is a problem the solution of which is to be found not by investigation of the gross means for the recognition of immunity and susceptibility but by study of the very source whence spring these states. The specificity indicated by immunity reactions is a property of the protoplasm of individual cells or their ground-substance. What is normal ground-substance ought to be so strictly determined that we can recognize this also for the human being. Once the range of normal variations is known, we may be able to determine how the protoplasm responds when disease enters. A pressing problem in medicine is thus the most exact recognition of states of health and disease as shown by the ground-substance of the cells. The poverty of methods of microscopy employed by medical workers need not satisfy us, however much these crude methods suffice for the needs of routine diagnosis.

To repeat: the inclusion-free substance of an egg is alone the living, the real, cytoplasm. When, therefore, we speak of the "structure" of cytoplasm we should adhere to this definition, dismissing thereby the alveolar, filar, granular, or other theory of protoplasmic structure which includes the entire cell contents. To postulate a plausible and practicable theory of the structure of cytoplasm we need to ascertain what the biological structure of the ground-substance is. Lack of this knowledge hinders not only biological conceptualization but also investigations by chemists and by physicists of the chemical structure and the physical attributes of the living substance.

II

Whatever the form of the protoplasmic system—without formed nucleus, or with one or more nuclei, be it of plants or of animals—it possesses surface-located cytoplasm. No protoplasmic system exists without an external limiting surface. Thus, the ectoplasm, an extension and differentiation of the ground-substance, is a *sine qua non* of living substance.

In bacteria with or without nuclear granules, the ectoplasm is a region qualitatively different from the inner core of the organism. The classes of Protozoa are most readily distinguished by their ectoplasmic structure. Thanks to Harrison, founder of the method of tissue culture, we possess valuable data concerning the physiology of the ectoplasm,

especially in the outgrowing nerve fiber. Viguer and others have extended our knowledge of the ectoplasm in tissue cells generally. For animal eggs I have reviewed observations on the occurrence of ectoplasm as found in every animal phylum.[6]

For egg cells we may note that the ectoplasm has a structure that is basically the same, namely, a series of projections of the surface cytoplasm inclosed by a fine membrane, product of the filaments. Upon this basic structure are imposed specific structural differences, such as length and thickness of the filaments and their disposition with respect to each other. In addition there are characteristics determined by the specific structure of the ground-substance of which the ectoplasm is a differentiated extension, displaying physical properties due to its peripheral location, which places it in direct contact with the cell's environment.

But it is the activity of the ectoplasm which constitutes its most prominent feature. It is the medium of entrance of gases, water, and foodstuffs from the external medium, and the gateway for the exit of gases, water, excretion, and secretion from the cell. Its role in such processes as fertilization, parthenogenesis, cell division, and differentiation is likewise clear. Thus, its role is not merely a passive one, owing to its peripheral location; it engages actively in the physiology of the cell. Moreover, it is the most readily prehensible cell region, revealing changes in normal processes easily susceptible to reversible experimental conditions and first affected by noxious treatment. Study of ectoplasmic activity is study of the most manifest, most easily tangible activity of differentiated ground-substance.

Whereas every protoplasmic system, cell or other, is a defined structure with a limiting boundary within whose confines the size of the system is set as a specific character, protoplasmic systems may or may not contain one or more discrete nuclei, as has been said above. Thus, the discrete nucleus is not a *sine qua non* of living substance. That the nucleus has specific structure is shown by the number, form, and size of the chromosomes, as well as by the form and size of the nucleus as a whole and by the structure of the mitotic spindle, when this is present. With the nucleic acid common to all plants and with that common to animals is conjugated a simple protein whose ensemble of amino acids makes the nucleoprotein a specific chemical compound. Because of chemical structure, the nucleus of every cell reveals physical attributes; presumably, therefore, these differ with different species.

In the developing egg there can be no doubt that nuclei are products of the ground-substance of the cytoplasm. The evidence supporting this proposition follows:

For those eggs upon which observations have been made, the ratio of volume of nuclear substance to cytoplasm steadily changes during cleavage, the total volume of nuclei increasing, the amount of cytoplasm decreasing. This statement holds for eggs of echinids, of mollusks, of nereids, and of *Amphioxus*. The measurements of nuclear increase should be begun at the time of first cleavage, because this is the first stage in which eggs are alike with respect to nuclear makeup, whether they are fertilized in the germinal-vesicle stage, during first maturation, during second maturation, or after maturation. The measurements ought be continued throughout the stages during which the eggs derive no food material from the environment; since eggs vary with respect to the time when yolk is transformed into hyaline ground-substance, and this depends upon the distribution of the yolk within the egg, the originally present hyaline ground-substance serves as a direct source for the nuclear increase for a period which differs with

[6] E. E. Just, *The Biology of the Cell Surface* (Philadelphia: Blakiston, 1939).

different eggs. These measurements end at the stage in which the developing egg derives food from its environment, be this nonliving or, as in the case of the mammalian egg, living. Obviously, with the ingestion and assimilation of foodstuffs, the synthesis of nucleoprotein, as that of other compounds, cannot be directly attributed to the original cell contents.

Eggs of echinids, of nereids, and of *Amphioxus* represent the simplest cases of nuclear growth at the expense of the ground-substance. Beginning with a ratio of nucleus to *total* extra-nuclear substance of about 1 to 1,000, the ratio steadily falls to around 1 to 3. As far as one can judge, the ratio is similar in the egg of *Crepidula;* at any rate, I have found that in the egg of *Aplysia* the increase of total nuclear content is of the same order as in these simplest cases. The difference between these and the molluscan eggs (of *Crepidula* and of *Aplysia*) lies in the fact that, in the latter, eggs with unequal cleavage, yolk is steadily being transformed into ground-substance during cleavage, whereas, in the former, the food reserves are used only later, i.e., after gastrulation. Eggs of cephalopods, arthropods, fishes, reptiles, and birds constitute the category of eggs with partial cleavage; in them the early utilization of yolk by the ground-substance is more marked. Finally, there are mammalian eggs, on the one side, and trematode and cestode eggs, on the other; in the first named, yolk is accumulated in the yolk sac, while in the second, cleavage is apart from the yolk whose origin is extrinsic to the egg.

Adding to the foregoing the fact that fragments of clear egg cytoplasm devoid of yolk synthesize nuclei, we reach the conclusion that nuclei are derivatives of the cytoplasmic ground-substance. Hence, nuclei are derivatives, differentiations, of their inclosing cytoplasmic ground-substance. This applies to all animal eggs.

This discussion of ectoplasm and of nucleus, derivatives of the cytoplasmic ground-substance, allows us now to define protoplasm: Living substance or protoplasm is the hyaline menstruum which pervades the entirety of the protoplasmic system and in which formed bodies are suspended; at its surface it shows always differentiated hyaline substance; often it contains one or more nuclei, differentiations of the hyaline menstruum.

III

What is meant by the "chemistry of protoplasm" depends, first of all, upon what is meant by "protoplasm." That which is to be subjected to chemical analysis ought not be needlessly burdened by substances additional to the material to be studied. No true chemistry of protoplasm, as defined above, has yet been made. Indeed, chemical investigations of protoplasm, even when this is considered as embracing the entire content of the protoplasmic system, cell or otherwise, have been embarrassed by the fact that the material analyzed has not always been free of contamination. This is especially true of tissue analysis, since with tissues most often are associated blood, body fluids, excretions, etc. The chemical analysis of single cells, demanding large numbers, similarly often includes the circumambient fluid in addition to the inert cell membranes no longer part of the true cells. A chemical analysis of protoplasm in our meaning is a chemical analysis of the ground-substance.

All that has been done chemically on protoplasmic systems *en gros* needs to be done on the ground-substance alone. Until such studies have been made, we have no chemistry of protoplasm but only a chemistry of the mixture within the boundaries of the system (and often including that of these boundaries). To obtain a conception of the chemistry of the ground-substance of cells from studies so far made, one may substract what is

known of the chemistry of the cytoplasmic inclusions and of that of the nuclei from what is known of the chemistry of the entire cell contents. It would be far better, however, to analyze directly the cytoplasm itself.

In view of the elegant researches recently made, and now under way, on the chemistry of proteins, thanks to the technique of X-ray spectroscopy and of ultracentrifuging, the reader may think my position too extreme in discounting the value of our present-day knowledge of the chemistry of protoplasm. Nevertheless, I maintain this position, for I do not see, despite the great advance in protein chemistry, that we have progressed very far beyond the stage reached when Delage wrote forty-five years ago. As a matter of fact, the work on protein chemistry serves pointedly to exemplify what, to me, is the greatest shortcoming of the chemistry of protoplasm, as usually defined.

Proteins in test tubes, brutalized by the procedures necessary for their analysis, are not the same as those in the living protoplasmic system. This being so, the beautiful researches on proteins by means of spectroscopy and ultracentrifuging inform us only of the structure of changed proteins. According to some workers, it makes no difference whether one works with denatured or nondenatured proteins. But, however slightly the protein may be changed, it is changed; and therefore it is something other than that in the intact protoplasmic system. If we could reconcile the various theories proffered today for the spatial structure of proteins—theories some of which are highly speculative—we should still need to reckon with the fact that proteins extracted from protoplasm are different from proteins in the viable protoplasmic system. Gelatin, the white of the hen's egg, and even the globulins and albumens of blood are extractives only: having been excreted, they are no longer part of the living protoplasm.

It is no escape from this situation to speak of "living" protein in this connection. Proteins exist as molecules; we have no evidence for the existence of life-molecules. If there are such, they are probably not protein only, since we have no knowledge of any life-thing composed of protein alone. Protoplasm, as matter in the living state, is a mélange in which react not only proteins but also carbohydrates, lipins, electrolytes, and, most abundantly, water. Therefore, not only should we avoid confusing protoplasmic extractives with protoplasm itself; we should also be wary of taking one protoplasmic component for the whole protoplasm. For any and all of the partners in the structure of protoplasm we need to recognize the differences between these as single entities when extracted for chemical analysis and these same in that close juxtaposition in which alone resides the living state. The physical and chemical properties of the single components in living protoplasm are not, of necessity, the same as the properties of these components in a mere physical mixture. The chemical activity of the components, when assembled as living protoplasm, probably differs even more from the activity of these components when taken singly than the activity of a molecule of lipin, of protein, or of polysaccharide differs from the activity of the constituent fatty acid and glycerol, the amino acid, or the simple sugar, respectively, when taken alone. Even the electrolytes, when separated, probably do not act as they do when in conjunction with each other and with other constituents in the protoplasmic system. Who knows whether water outside a cell has precisely the same properties as water within it? Here, again, one does not avoid the problem by speaking of "living" water, a term which means nothing as long as we do not know what life is. Until we possess this knowledge, it is idle to seek an answer to this perennial riddle by hypothecating to single protoplasmic constituents, removed from their union, the life-principle which, so far as we

know, resides only in the whole, intact protoplasmic system. What is said of the proteins isolated from the intact cell, or other form of protoplasm, may be said of any component of it removed from its normal combination. Thus, here pure chemical analysis defeats itself by virtue of its purity: living protoplasm is not, in this sense, "pure."

However, to close up the gap in our knowledge of the chemistry of protoplasm, we should not merely carry over to the study of the ground-substance methods utilized heretofore on the whole protoplasmic contents. We need, rather, to adopt methods applicable to the living protoplasm itself and devised for it, however difficult it may be to place ourselves in a new way. Indications are not wanting to show that it is possible to study the chemistry of ground-substance directly in the living condition, although at present the methods are, of necessity, crude and only qualitative.

A word about enzymes may not be amiss here. Work such as that by Holter on the localization of enzymes within cells shows that the ground-substance alone contains them. Extension of this work will doubtless give clues of value concerning the chemical composition of living protoplasm.

The enzymes best known chemically are either simple proteins, such as pepsin and trypsin, or conjugated proteins, as, for example, the respiratory ferment (oxidative catalyst). If it should prove to be true that all enzymes are proteins, we can say that the intraprotoplasmic chemical reactions characteristic of living substance probably are due to some peculiar protein bodies. Adding that bacteriophage is perhaps a conjugated nucleoprotein and that an ultrafilterable virus isolated by methods used for the purification of pepsin and trypsin appears to be an enzyme, we conclude that certain proteins play a definite role in intraprotoplasmic reactions. These reactions, however, involve other compounds than proteins, since not all the chemically known enzymes are proteolytic; they break down (and build up) other constituents—carbohydrates, fats, and still simpler compounds—and hasten oxidation. Thus, urease is not a proteolytic enzyme. Undoubtedly many intraprotoplasmic reactions are autocatalytic; as such, they demand precursor reactions, since they do not start of themselves. The study of autocatalysis in living systems is therefore strongly indicated. One such lies close at hand, the synthesis of nucleoprotein, as revealed by the increase of nuclei during the early stages of an egg's development. In the cytoplasmic ground-substance reside the precursors of the nucleoprotein which constitutes the nuclei.

Enzymes act singly or with coenzymes to break down or to build up compounds. Some, such as pepsin, trypsin, etc., are extracellular, acting after having been excreted by the protoplasm; others, the intracellular, offer more difficulty for study. Until more is known of the latter, comparison of their activity with that of extracellular ones is hazardous. Much of the work on intracellular enzymes I consider doubtful, for the reason that it is done on the enzymes after they have been isolated, the protoplasm having been destroyed. Such work on intracellular enzymes concerns itself with the presence of coenzymes, the speed of enzyme reaction, and the quantity of enzymes present. With respect to the first, the methods of isolation may induce formations interpreted as coenzymes. Further, the speed of reaction within a cell and in a test tube may be different. Up until now, our ignorance of the underlying physical conditions which contribute to the velocity of protoplasmic reactions is too great to warrant an assumption as to the identity of speed of reaction in living protoplasm and in test tubes. Finally, the quantity of enzyme present does not always give the rate of reaction or indicate that reaction takes place.

It cannot be overemphasized that what we need in the chemical study of the ground-substance is chemical analysis not only of the dead stuff but also of the living. The latter is difficult but not impossible. Such chemical study should include, first, the mapping-out of the pattern of the ground-substance specific for each protoplasmic system. Animal eggs furnish excellent objects for this type of investigation.

Results of my own, as yet unpublished in detail, belong here. By means of staining and of precipitation reactions I have been able to relate the intimate structure of the ground-substance to the specific organization of a given egg, as well as to the changes in a named species of egg that occur during the mitotic cycle. Spectroscopic investigation of the ground-substance is also possible, as I have found.

By means of studying the dispersion, appearance, and disappearance of water drops in cells I have been able to obtain a clue concerning some enzyme reactions in living, viable egg cells. This, an indirect method to be sure, is excellent for investigating hydrolysis and synthesis occurring within the cytoplasm. With it I have been able to study, for example, the hydrolysis and synthesis of lipin in living endoderm cells, without disturbing in any wise the normal processes of development. Further, the appearance and disappearance of water drops in perfectly normal cells furnishes a reliable index for the onset and direction of rhythmical protoplasmic behavior.

The progressive synthesis of the nucleoprotein, as this is shown in eggs during cleavage by the progressive increase of nuclear material, also demands our attention.

The studies made with the Feulgen reaction and those made by Caspersson are aids in the investigation of nuclear material. The methods of Caspersson and similar ones could be so extended that they would throw light on those reactions in the ground-substance antecedent to the synthesis of nucleoprotein. These, in combination with the use of the old Altmann method, as revived by Bensley and Gersh, promise much. No problem in the whole field of cellular biology carries more weight than this of the progressive building-up of nuclear matter out of ground-substance. A parallel investigation ought to be made on the growth and reproduction of noncellular protoplasts.

What has been said above concerning the biological (morphological and physiological) structure of protoplasm, as well as concerning its chemical makeup, applies equally to its physical structure. Physical studies on an entire protoplasmic system, including effete substances, food material, etc.; such studies as those on permeability; or others on single properties of single regions of the cell may have value. But there is an urgent need for the study of the physical properties of protoplasm per se, of the living ground-substance. Here, also, study of the rhythmical appearance and disappearance of water drops in normal cells would be of incalculable help. The too great neglect of investigation of the behavior of water, quantitatively the most abundant compound in living substance, amounts to a reproach. As long as we remain in ignorance concerning the distribution of water in the normal protoplasmic system, our theories of the physics of the living state rest, of necessity, on an incomplete foundation.

Moreover, the sooner we dismiss the theory of surface tension in biology, together with the theories of permeability, the earlier we shall approach a substantial knowledge of the physical structure of protoplasm. Surface tension, it seems, is a chemical phenomenon. At any rate, it applies to molecules. From this point of view alone, it would seem to be as dangerous to apply its use to protoplasmic systems as to electrons: the former are too complex, the latter not complex enough. One only begs the question when one speaks of "tension at the surface." Theories both of permeability and of surface-

tension in biology ignore the ectoplasmic structure of the protoplasmic system; overlooking the livingness of living things, the authors of these theories accumulate evidence from elegant experiments on nonliving membranes and inert powders spread upon water.

For biological, as well as for chemical and physical, investigations, the first question to be answered is: What is normal living protoplasm? The failure to recognize this normality may be designated as the chief characteristic of many of the current researches in chemical and physical biology. The orthodox biologist also is often guilty of being ignorant of the normal process in the system under investigation. Because of the fact that experimentalists frequently do not appreciate normal processes, their experiments, especially those that debase the normal state, often yield results of little significance for the object of the work, an explanation of normal phenomena. These processes and their range of normal variation must be fully recognized before an attempt can be made by methods of experimental pathology to interpret them. A serious responsibility rests upon the descriptive biologist to establish, beyond question, criteria of normality, the range of normal processes, and the extent of their normal variability. A physics and a chemistry of living protoplasm worthy of being so called depend upon this descriptive biology.

IV

The problems of general biology named at the outset hang upon the problem of the biological, chemical, and physical structure of protoplasm. Concerning the origin of life, the maintenance of its specific form, and its specific reduplication, we may speculate—have speculated—without having been able to place these questions in the category of those fully answered. Modern theories proffered to explain these problems of general biology ignore, if they do not discount, the basic problem of the structure of protoplasm.

These theories rest, in turn, upon one or the other of two conceptions which have dominated biological thinking and provoked various expressions of allegiance on the part of their respective adherents. I refer to the mechanistic (nowadays often denominated the physicochemical) and the vitalistic schools. In my judgment, the fight between mechanism and vitalism is in large measure a war of words, as I have elsewhere pointed out. But, if for the moment we take these terms as truly representing a joined issue, we discover yet another fallacy, which amounts to a defection in the camp whose aegis is mechanism.

In attempts at mechanistic interpretations the conception is only allegedly mechanistic, and in reality is as vitalistic as any statement of purest vitalism.

With the concept of evolution the case is clear. Evolution is a concept pure and simple, and not a demonstrated scientific truth, however much the evidence in its favor is accepted. Mechanistic explanations fail here. Such terms as "survival of the fittest," "struggle for existence," "natural selection," and the like owe their power as much to their poetical connotation as to their biological basis. Biology, we must recognize, is a welter of such figurative expressions and of physical and chemical borrowings that obtrude themselves irritatingly whenever we attempt to precise the meanings of words employed in the science—indeed, their too abundant use often tends to place the science outside the realm of other natural sciences, rendering difficult exactitude of expression. To be sure, chemistry uses terms such as "chemical affinity," and physics such as "attraction," for example; but usage in the physical sciences has sharpened the meaning of terms, whereas in biology comparable ones carry varying imports.

Take, as another example, the concept of the axial, polar, physiological, metabolic gradient, or of physiological dominance of fields—call it what you will. These terms are merely pretentions. They seem to be physical but are only the mechanistic biologists' euphemisms for "vital force," "entelechy," "*élan vital*," etc., inasmuch as they substitute for expressions avowedly vitalistic a new term which differs only in so far as it means that life-processes are organized along an axis instead of being not so limited by the upholders of the theories of entelechy and the like.

Or, consider the concept of the gene. Many a sufferer of typhoid fever or of another infectious bacterial disease knows, to his discomfort, that bacteria hand on their heritage. Most bacteria possess no chromosomes. The gene theory does not hold for such organisms. Indeed, the theory may or may not hold for organisms containing nuclei and chromosomes. But, even if it could be proved to hold for every single living thing, plant and animal, it is far from being the physical concept claimed for it by its founder, i.e., that it is a concept built upon quantitative experience as elegant as the beautiful work on the electron. Its mathematical basis is far removed from the exquisite fineness of modern physical measurements. The gene theory, however much it pretends to be mechanistic, is only crudely vitalistic: it gives us a set of demons, the genes, capable of extricating the theorists from every difficult situation. For one entelechy it sets up many.

These alleged "mechanistic" interpretations have failed. They tell us, though covertly, that to formulate life we need to invoke terms, as "entelechy," "vital principle," "*élan vital*." The mechanists should, therefore, abandon synonymous expressions no less vital than these, because they are garbled physical concepts. There remains the way of investigating life from a *nonmechanistic* point of view, which is, however, in no sense vitalistic.

Life is not a metaphysical vagary but a phenomenon which we do not yet comprehend. The practicable point of view which, it seems to me, is most productive is that which concentrates on the visible manifestations of livingness, the tangible expressions, the phenomena. Once the phenomena are apprehended, the right conception will emerge. Certainly, up to now, our conceptions leave much to be desired.

Not less inept than these opposing mechanistic and vitalistic conceptions are the theories underlying the three above-named problems of general biology.

Certainly, nowadays no one holds to the outmoded view that study of fertilization and of experimental parthenogenesis can aid us directly in discovering how life arose. This view never would have prevailed but for the ardent faith of blind worshipers who saw in the early work on experimental parthenogenesis the creation of life. It may be that life *was* created—if so, then we be futile laborers who seek to unravel the mystery of its origin and evolution. But, if life arose and evolved, then the understanding of its origin and evolution again wait upon further knowledge of protoplasmic structure. What arose and what has evolved are the insisting and persisting questions. They concern the living substance par excellence, i.e., the ground-substance.

A point less disputed perhaps, but none the less important, is that of the ordered maintenance of life-forms persisting through generations. What is maintained—what persists? Here, again, the fundamental question is that of protoplasmic structure. As in every single domain of cytological investigation known to me, we see here darkly through a glass clouded by the haze of bad definition. Growth is often confounded with differentiation and—in the case of cells and especially of egg cells—with cell division. The self-regulation, self-maintenance, and self-perpetuation of the protoplasmic system

constitute phenomena of paramount importance. With heredity and evolution they are inextricably bound: they, too, have been inherited, have evolved.

To whatever extent we accept the data of geneticists, we are loathe to regard, as does the founder of the gene theory, the problem of heredity as solved. On the contrary. Until we know much more concerning the biological, chemical, and physical structure of protoplasm, the problem of heredity remains open. A theory of heredity must apply to all animals and all plants, whether these are composed of nucleated protoplasm or not. For beings made up of cells, we must know, beyond question, the origin and synthesis out of the ground-substance of the genes constituting the chromosomes in nuclei. Moreover, we must know how, upon the thread of the nucleic acid common to plants and that common to animals, the specific gene entities are imposed—unless the genes are nucleic acid. What knowledge we possess up to now only allows us to consider the chromosomes as indicators of deeper-seated processes.

In the preceding pages I have endeavored to show that the pressing problem in general biology is a clear recognition of what in any protoplasmic system, cell or otherwise, is the living substance. In default of this recognition, the questions of how life begins, how it is continued, and how transmitted will remain unanswered. Thus, knowledge of protoplasmic structure is basic for these, as yet unsolved, problems of general biology. To obtain a conception of this structure demands a concerted attack by biologists, chemists, and physicists who appreciate the limitations of the life-state, the range of normal life-processes. Said briefly, we need a biology that recognizes its field and the problems peculiar to it and which treats these according to its domain and what that domain embraces.

PHYSIOLOGY OF DEVELOPMENT OF THE FEATHER. III GROWTH OF THE MESODERMAL CONSTITUENTS AND BLOOD CIRCULATION IN THE PULP[1]

(Nine text figures; four plates)

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I. INTRODUCTION

THE following investigation started out from the fact that in studies of the development of the feather attention has been directed almost exclusively to the ectodermal constituents; this was also the case in our own previous studies (Lillie and Juhn, 1932, 1938). The problems of the growth and functioning of the mesodermal constituents, pulp and papilla, of the feather germ have thus been relatively neglected.

All growth of the feather proper is dependent on the blood supply of the pulp, which bears the entire burden of nutrition; all hormonal influences affecting the form or pattern of the feather are transmitted by the blood; other chemical variations in the blood stream and variations of blood pressure must also play their part in the development of the feather.

Moreover, there does not exist anywhere in the animal kingdom, so far as I know, such extensive vascular protrusions from the surface of the body as in the pulps of

[1] The expenses of this investigation were supported in part by a grant from the Rockefeller Foundation to the University of Chicago.

growing feathers, which reach a length of at least 6 cm. in wing and tail feathers of the fowl, and probably even greater lengths in some larger birds. What, then, are the special vascular arrangements that control all this conspicuous functioning?

Such questions, with their obvious reference to our preceding investigations, furnished the starting-point and motive for the study that follows. They soon led into inseparable problems of growth.

The White Leghorn fowl has been used, for the most part, in this study, because the absence of chromatophores and pigment is a great advantage in studying the circulation; but Brown Leghorns have also been used to some extent. The adult was used generally, but occasionally the juvenile plumage was used.

The injections, the measurements, and the technical operations were performed by my research assistant, Mr. Hsi Wang, under general direction. To his skill and devotion to the work the writer is deeply indebted. The microscopic sections were made by Miss Margaret Davidson, of the General Biological Supply House. Reference is made to Figures 18 and 19 for general topography.

II. THE PULP AND THE PAPILLA (GENERAL)

The pulp is a loose mesenchymal reticulum, the interstices of which are occupied mainly by a homogeneous, gelatinous substance that gives the entire pulp a firm, tough, and elastic consistency. A colorless serum also exudes from injured surfaces of the pulp. The rich, highly complex vascular system of the pulp is described in section IV.

The pulp exerts considerable pressure on the ectodermal cylinder of the feather germ and thus keeps it in a state of tension which is responsible, among other things, for the smooth, even surface of the sheath. If a plucked regenerating feather is kept in the open air, the sheath soon wrinkles, owing to loss of water from the pulp; but the sheath remains smooth indefinitely in a moist chamber. The sheath normally persists several millimeters above the apex of the pulp. Turgor of the pulp[2] can readily be demonstrated by removing the sheath of a regenerating breast feather down to the mouth of the follicle with the feather *in situ* in the living bird. The exposed barbs are then unable to resist the internal pressure of the pulp, which expands and forms a swelling, tapering in an apical direction, just above the mouth of the follicle. This happens in a very few minutes. If 2–4 mm. of the sheath is left above the mouth of the follicle, a similar, but smaller, swelling develops above the sheath. The apical third of the pulp does not swell so readily, owing to increasing thickness of the pulp membrane (p. 153).

No evidences of cell division are found anywhere in the pulp itself. The cellular richness of the pulp is greatest next to the papilla[3] (Pl. II, Fig. 1), and rapidly diminishes, with corresponding increase of intercellular substance, in an apical direction, and then remains practically uniform to the apex (cf. Davies, 1889, and Lillie and Juhn, 1932). The pulp, in fact, like the feather proper, grows from its base; and there can be no doubt that the cells are derived from the papilla.

The pulp arises from the papilla at the onset of regeneration of the feather and is

[2] The problem of turgor is discussed later; see pp. 172–73.

[3] The term "papilla" is used in this paper exclusively for the persistent dermal structure underlying the pulp and filling the opening of the umbilicus. Davies (1889, p. 572 n.) used the term as synonymous with "feather germ," i.e., as including both epidermal and dermal constituents. He used the term "pulp" to include also the papilla in our sense.

finally completely resorbed when the feather is fully formed. If the growing feather is plucked, the pulp comes away, but the papilla remains.

In the papilla, apical, basal, and peripheral surfaces may be distinguished. Bundles of interwoven fibers give it a tough consistency, especially in its basal half. It is constricted about midway between apical and basal surfaces by the lip of the umbilicus (collar) (Pl. II, Fig. 1), so as to have an hourglass form.

The basal half is cleanly delimited from the derma over its hemispherical boundary, and a strong bundle of fibers is prolonged around the periphery of the base into the wall of the follicle. It is thus firmly anchored. The apical surface forms a sharper dome, the center of which may be raised around the axial artery. The periphery of the apical half sends a prolongation along the inner surface of the collar.

The apical surface of the papilla, in contrast to the basal surface, is not sharply delimited. On the contrary, it is frayed out into strands of cells that merge with the pulp. This is the region of greatest cellular richness of the pulp referred to above. This contribution to the pulp from the papilla involves not only the arched surface of the apex but also the periphery which extends along the collar, as shown in Plate II, Figure 1.

From the time of its formation the pulp is most intimately associated with the surface of the collar, though separated by the basement membrane from the epithelial cells of the collar. In the region of the ramogenous zone the basement membrane thins out and may even seem to disappear. This is a very narrow circular zone interrupted dorsally by the shaft in which the organization and cellular growth of the ridges occurs, and along which the tangential movement of the bases of the ridges is directed toward the shaft (Lillie and Juhn, 1938).

In addition to its functions with reference to the pulp, the papilla has certain morphogenetic functions. This is to be inferred by its persistence as the locus of development, replacement, and regeneration of feathers from the time of its origin in the embryo throughout life; but it is not known what morphogenetic relations actually exist between the papilla and the ectodermal component of the feather germ, and this subject is not considered in the present paper.

III. GROWTH OF THE PULP DURING REGENERATION

1. GROWTH IN LENGTH

The growth in length of the entire feather from the thirteenth day of regeneration until completion was measured at 2-day intervals for the anterior part of the breast, for the posterior part, and for saddle feathers. The length of the pulp was also ascertained for each measurement, and the percentage of the pulp length to the total length was then calculated.

The measurements for the breast feathers were made on a White Leghorn cock, which possesses the advantage that the vascular pulp can be seen *in vivo* and the same feathers and their pulps can be measured during the entire period of regeneration. The measurements given in Tables 1 and 2 are the averages of the measurements of three adjacent regenerating feathers.[4] The data in Table 2 are shown in the form of curves (Fig. 2).

[4] The measurements of length of the living feathers are, of course, taken from the mouth of the follicle. In order to obtain the length within the follicle, which is added, a neighboring regenerating feather of the same length as the average of the three measured *in vivo* was plucked and the length within the follicle ascertained. This is a quite constant quantity. The plucked feather was also used for determination of weight of constituents (see p. 166).

In the case of the saddle feathers, measurements were taken on Brown Leghorn capons in which the vascular pulp cannot be seen *in vivo*. This necessitated the plucking of a large area and the sacrifice of five regenerating feathers actually plucked for each

TABLE 1

WHITE LEGHORN COCK: ANTERIOR BREAST FEATHER*

Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)†	Percentage of Pulp Length to Total Length	Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)†	Percentage of Pulp Length to Total Length
13	16.0	11.7	73.1	34	58.7	12.7	21.6
14	17.2	12.0	69.8	36	63.0	13.0	20.6
16	21.7	12.7	58.5	38	66.0	10.8	16.4
18	25.3	12.0	47.4	40	70.0	10.7	15.3
20	29.7	11.7	39.4	42	73.0	10.3	14.1
22	34.0	11.3	33.2	44	75.0	8.5	11.3
24	37.0	11.5	31.1	46	76.0	7.0	9.2
26	43.0	12.0	27.9	48	77.3	5.0	6.5
28	48.0	12.0	25.0	50	79.0	3.0	3.8
30	50.7	12.5	24.7	52	79.0	2.0	2.5
32	55.3	12.8	23.1	54	79.0	0.0	0.0

* Length of pulp in relation to total length of regenerating feather, measured *in vivo*. On account of absence of pigment, the pulp can be seen *in situ*. The measurements are the average of three figures. The lengths (measured from the skin) are corrected by adding the depth of the follicle ascertained by plucking control feathers.

† The sum of all measurements of length of pulp, amounting to 215.2 mm., is referred to on p. 166.

TABLE 2

WHITE LEGHORN COCK: POSTERIOR BREAST FEATHER*

Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)†	Percentage of Pulp Length to Total Length	Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)	Percentage of Pulp Length to Total Length
13	18.5	12.7	68.6	38	81.0	13.0	16.0
14	21.0	13.5	64.3	40	85.0	12.2	14.4
16	25.7	14.5	56.4	42	88.7	12.0	13.5
18	31.0	15.0	48.4	44	92.7	12.0	13.0
20	35.7	15.2	42.6	46	96.7	12.0	12.4
22	41.0	15.2	37.0	48	100.3	11.0	11.0
24	46.0	15.0	32.6	50	103.0	9.0	8.7
26	51.2	14.5	28.3	52	105.0	6.5	6.2
28	56.3	14.0	24.9	54	106.0	4.0	3.8
30	61.2	14.0	22.9	56	107.0	3.0	2.8
32	66.0	14.0	21.2	58	108.0	2.0	1.9
34	71.0	13.7	19.3	60	108.5	1.0	1.0
36	76.0	13.5	17.8	62	108.5	0.0	0.0

* Length of pulp in relation to total length of regenerating feather, measured *in vivo*. On account of absence of pigment, the pulp can be seen *in situ*. The measurements are the average of three figures. The lengths (measured from the skin) are corrected by adding the depth of the follicle ascertained by plucking control feathers.

entry, and dissected so as to show the length of the pulp. This has the disadvantage of introducing irregularities due to different locations in the same tract. The results of measurements on two birds, as given in Tables 3 and 4 and in the curves (Fig. 3) based

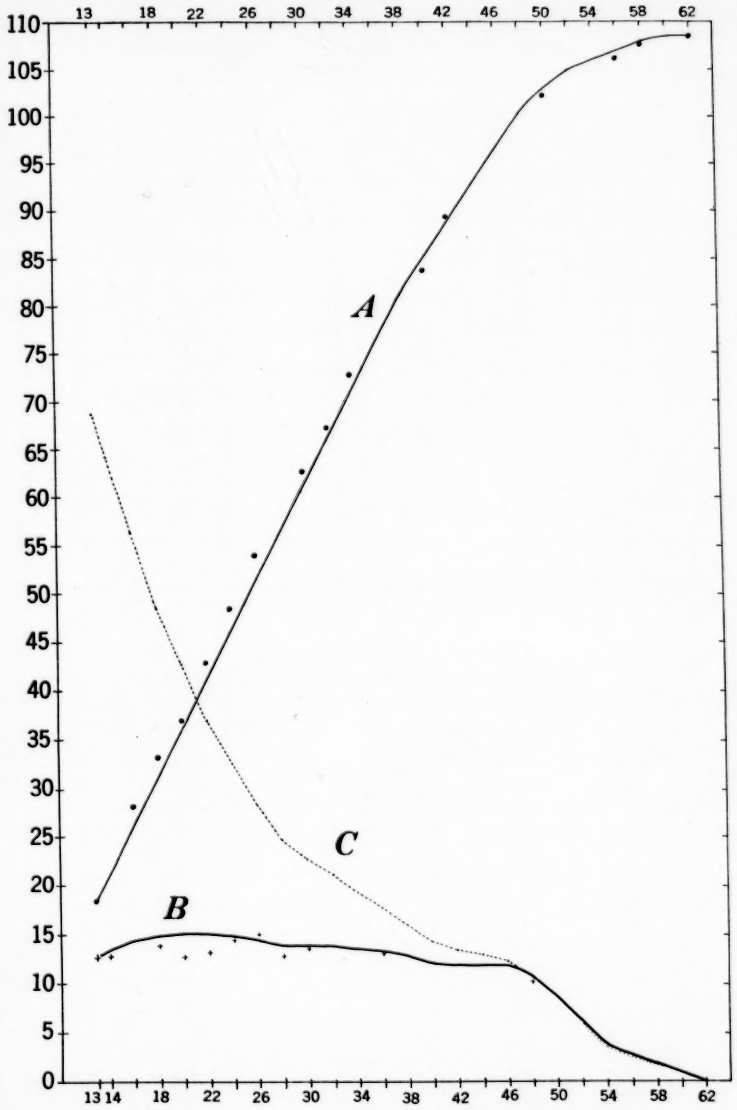

FIG. 2.—Curves of growth, feather and pulp, posterior breast feather, White Leghorn cock. Data from Table 2. *A* = feather; *B* = pulp; *C* = percentage of length of pulp to length of entire feather. Abscissae = days of regeneration; ordinates = length in millimeters for curves *A* and *B*, percentages for curve *C*.

TABLE 3

BROWN LEGHORN CAPON 171: SADDLE FEATHER*

Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)†	Percentage of Pulp Length to Total Length	Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)†	Percentage of Pulp Length to Total Length
14	12.5	11.5	92.0	51	79.3	13.0	16.4
16	17.0	13.7	80.6	53	82.7	12.5	15.1
18	19.7	14.8	75.1	55	86.5	12.3	14.2
20	23.4	15.2	64.9	57	90.0	12.2	13.6
22	27.0	15.3	56.7	59	93.0	12.1	13.0
24	30.5	14.8	48.5	61	96.2	12.0	12.3
26	34.3	14.2	41.4	63	99.5	12.0	12.0
28	38.0	13.4	35.3	65	102.5	12.0	11.7
30	41.5	13.2	31.8	67	105.5	12.2	11.6
32	45.0	13.0	29.0	69	108.7	12.3	11.3
33	47.0	12.8	27.3	71	112.0	12.5	11.2
35	50.5	12.8	25.3	73	115.0	12.7	11.0
37	54.0	12.9	23.9	75	118.0	12.0	10.2
39	57.5	13.0	22.6	77	120.2	10.7	8.9
41	61.0	13.1	21.5	79	122.5	8.7	7.1
43	65.0	13.4	20.6	81	125.0	6.2	5.0
45	68.5	13.7	20.0	83	127.0	3.0	2.4
47	72.0	13.6	18.9	85	129.0	0.0	0.0
49	75.5	13.5	17.9				

* Length of pulp in relation to total length. The measurements are the average of 5 plucked feathers for each entry.
† The sum of all measurements of length of pulp, amounting to 443.3 mm., is referred to on p. 165.

TABLE 4

BROWN LEGHORN CAPON 148: SADDLE FEATHER*

Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)†	Percentage of Pulp Length to Total Length	Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)†	Percentage of Pulp Length to Total Length
13	11.5	10.5	91.3	49	78.0	13.0	16.7
15	15.5	14.0	90.3	51	81.5	12.5	15.3
17	19.0	15.5	81.6	53	85.5	12.2	14.3
19	20.5	15.2	74.1	55	89.0	11.8	13.3
21	26.5	14.8	55.8	57	93.0	11.5	12.4
23	30.5	14.4	47.2	59	96.5	11.2	11.6
25	34.0	14.0	41.2	61	97.5	11.3	11.6
27	37.5	13.8	36.8	63	100.5	11.5	11.4
29	42.5	13.5	31.8	65	103.0	12.0	11.7
31	45.0	13.2	29.3	67	105.0	12.5	11.9
33	48.5	13.2	27.2	69	107.5	13.0	12.1
35	52.5	13.5	25.7	71	109.5	13.0	11.9
37	56.0	13.7	24.5	73	111.5	12.0	10.8
39	60.0	13.7	22.8	75	114.0	10.2	9.0
41	63.5	13.7	21.6	77	115.5	8.2	7.1
43	67.0	13.5	20.2	79	116.5	5.5	4.7
45	70.5	13.3	18.9	81	118.0	2.5	2.1
47	74.5	13.2	17.7	83	119.5	0.0	0.0

* Length of pulp in relation to total length. The measurements are the average of 5 plucked feathers for each entry.
† The sum of all measurements of length of pulp, amounting to 430.6 mm., is referred to on p. 165.

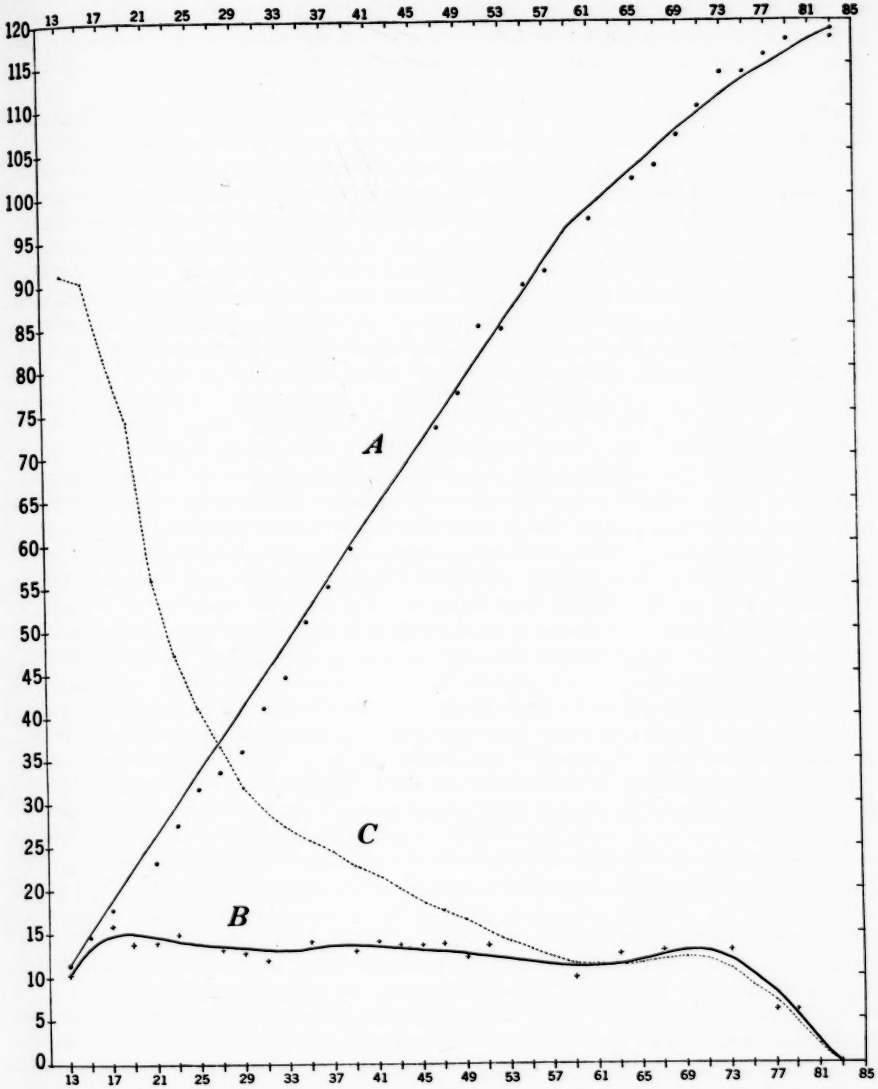

FIG. 3.—Curves of growth, feather and pulp, saddle feather, Brown Leghorn capon 148. Data from Table 4. *A* = feather; *B* = pulp; *C* = percentage of length of pulp to length of entire feather. Abscissae = days of regeneration; ordinates = length in millimeters for curves *A* and *B*, percentages for curve *C*.

on Table 4, however, agree very well with one another and are the same in principle as for the breast feathers of the White Leghorn. It will be noted that the measurements begin on the thirteenth or the fourteenth day of regeneration. The reasons for this will appear later.

The curves of growth of the entire feather do not differ significantly from previous determinations (Juhn, Faulkner, and Gustavson, 1931; Lillie and Juhn, 1932). The curves of length of the pulp and of percentage of length of pulp to the entire feather are, however, new determinations. The outstanding feature is that the pulp attains a certain "functional" length at about the fourteenth day of regeneration in the case of the breast feather, and a little later in the case of the saddle feather, which is maintained with only slight fluctuations throughout most of the period of regeneration. In the last 10–12 days of regeneration (cf. tables and curves for details) the length of the pulp decreases rapidly until it is all gone. This coincides with the period of most rapid decrease of rate of growth of the entire feather, and agrees approximately with the period of formation of the calamus of the feather.

2. FIXED RELATIONS OF PULP AND FEATHER

Considered entirely by themselves, these figures and curves, especially the long "plateau" in the case of the length of the pulp, might suggest that the ectodermal component of the cylindrical growing part of the feather is constantly slipping over the pulp in its apparently more rapid growth. The consideration that this would involve constant disturbance of delicate nutritive relations suggests doubts which are reinforced by the intimacy of relations between the pulp and the growing part of the feather. The relationship of the pulp to the ectodermal cylinder is so close that it is difficult to strip the pulp from the opened cylinder without carrying with it parts of the ridges, containing large melanophores in the case of black feathers. This is true in certain breeds of fowl, at least.

The alternative theory would be that the relations between the pulp and all formed loci of the ridges are permanently fixed throughout growth; that the pulp accordingly grows at the same rate, to the same extent, and in the same dimensions as the ectodermal component; and, therefore, that the total growth in length of the pulp during regeneration is equal to that of the feather itself. From this it would follow that the functional length of the pulp, as given in the curves, is regulated by resorption at its apex, where it becomes free from the shaft and barbs of the growing feather. We discussed these relations previously (Lillie and Juhn, 1932, pp. 145–46) and concluded: "We must thus think of the pulp as constantly growing at its base, and constantly dying and being resorbed at its apex."

We have now tested these hypotheses experimentally, and also by study of the process of resorption which results in the formation of the "pulp caps."

During a period of 7 weeks about 100 regenerating breast feathers of Brown and also of White Leghorns were operated on as follows, and 77 useful results were obtained. The procedure was to perforate a growing feather of from 21 to 28 days' regeneration transversely by a fine wire; in some cases the ends were cut off short and a piece left in; in others the wire was used to pull through a delicate silk thread, stained or unstained, a piece of which was similarly left *in situ;* finally, in yet other cases, a thread stained in gelatin carmine or Janus green was drawn completely through, leaving a stained track. At the stage of operation the pulp is 14–15 mm. long, approximately

8 mm. of which is above skin level. The perforation was made at skin level, and the track passed through sheath and barbs, through the center of the pulp, and out on the opposite side. Most of the operated feathers survived and grew at a normal rate, except for a possible brief period of inhibition following the operation.

The point to be ascertained was whether the pulp grew at the same rate as barbs and sheath—especially, of course, with reference to maintenance of fixed relations between the periphery of the pulp and the ectoderm. The experiments were uniformly convincing on this point. The mark on the periphery of the pulp and the external scar were always in perfect alignment.

The first experiments were with an exceedingly fine wire, left in place with free ends on the two sides. These experiments were exploratory with a view to ascertaining the possibility of the method. Objection could be raised to the use of the wire left in place on account of its rigidity. However, there was never any evidence, on careful examination by dissection, of strain in the pulp, such as would be expected if the rate of growth of the pulp were markedly different from that of the ectodermal cylinder. Seventeen feathers were so operated, and observations were made on different feathers 24, 48, and 72 hours after insertion. The rate of growth of the entire feather was 2.5–3.0 mm. per day, and the wire moved an equal distance above the surface of the skin; thus, after 3 days, as the pulp maintains an approximately equal length during this period, the wire was near the tip of the pulp, or beyond it, in a forming pulp cap. In all of the cases in which the wire perforated both sides of the sheath there was no evidence on dissection of abnormal relations of pulp and feather. Both appeared to have grown at the same rate.

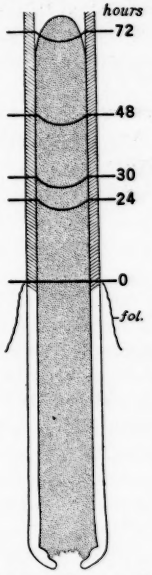

FIG. 6.—Diagram of path of track made by a silk thread left *in situ* from the time of its insertion through the center of the pulp. At the surface of the skin = 0; 24, 30, 48, and 72 show the position and the form of the track at 24, 30, 48, and 72 hours after insertion. × *ca* 9.

In some cases in which the wire was purposely not pushed through the opposite side, the free end in the pulp was at a slightly lower level than the opposite end perforating the sheath, thus, as a whole, being in a slightly slanting position. This suggested that the center of the pulp might be growing at a slightly slower rate than the periphery. It was, however, noticeable that the slant was as pronounced 24 hours after insertion of the wire as it was later.

The remaining experiments with fine silk threads or stained tracks were made to test the results with the wire left in place. Without exception they demonstrate that the surface of the pulp grows at the same rate as the sheath and barbs, for the points of entry on both sides exactly correspond even after 48–72 hours' growth (Fig. 6). The conclusion is then unescapable that the relation of the pulp and ectodermal component is a fixed one, at least for the portion above the skin. The same conditions must hold also for the portions yet in the follicle, which are constantly emerging from it as growth progresses.

The apparent slight difference between growth in the center of the pulp and at the

periphery remains to be explained. The course of the fine silk thread or of the stained track in the pulp is usually somewhat curved downward from periphery to center of pulp; but this is variable, and the path is sometimes approximately straight. Moreover, the curve is not more pronounced at 48 or 72 hours after insertion than at 24 hours (Fig. 6). The insertion of the wire of course ruptures some of the delicate-walled blood vessels of the pulp, and there may be slight external bleeding. The large vessels, especially the axial artery, are toward the center of the pulp, and hence the greatest injury is here. The evidence indicates that the downward curve in the track is an injury phenomenon, and that, once repaired, growth is equal from center to periphery of the pulp.

These experiments also demonstrate the occurrence of resorption at the apex of the pulp. Any transfixed level of the pulp becomes apical in the course of growth, and then disappears, leaving the transfixing wire or thread free above it. Thereafter the wire or thread continues to be carried upward by the continued growth of sheath and barbs.

3. RESORPTION AND THE PULP CAPS

A. INTRODUCTION

The resorption of the pulp takes place apically; it follows from the principle of equal growth of pulp and feather that the total amount of resorption is measured in length at any time recorded in the curves by the vertical distance that separates the curve of length of the pulp and of the entire feather (Figs. 2 and 3).

Before about the fifteenth day of regeneration typical pulp caps are not formed, and the length of the pulp is constantly increasing.[5] Thereafter the amount of resorption is approximately equal to the growth, as shown by the plateau form of the curve of length of the pulp for a long time during regeneration; finally, resorption overbalances growth, and the entire pulp disappears with the completion of regeneration.[6] The actual length of pulp at any time depends upon the relation between growth and resorption. The capillary system of the pulp is specially developed apically in connection with resorption (see sec. IV).

The growth of the pulp is continuous, but resorption is periodic and accompanied, after about the fifteenth day, by the formation of "pulp caps" at the rate of about one per day. In order to understand this process we must first consider the ectoderm membrane (stratum cylindricum) that separates the pulp from the formed elements of the feather.

B. THE STRATUM CYLINDRICUM

The ectoderm of the feather germ early becomes differentiated into three layers (Pl. I, Figs. 4 and 5): an outer layer (stratum corneum) next to the cavity of the follicle from which the sheath is derived; a thick middle layer (stratum intermedium)

[5] The formation of pulp caps is always associated with resorption of pulp, as described below. In the case of breast feathers, the first pulp cap is usually found on the sixteenth day of regeneration and only exceptionally as early as the fifteenth day. In the case of saddle feathers, the first pulp cap is formed between the fourteenth and sixteenth days. For earlier history of the pulp and the beginning of resorption see sec. III, 4.

[6] The rate of resorption is, however, not greatest at the end. Between the twenty-sixth and twenty-eighth days of regeneration in the case of the anterior breast feathers, 5 mm. of pulp was resorbed during the plateau period; between the forty-second and forty-fourth days during the period of diminution in length of the pulp, only 2 mm. of pulp was resorbed (see Table 1).

from which the entire feather—barbs, barbules, and shaft—is derived; and an inner epithelial layer (stratum cylindricum), next to the pulp. These layers are united basally in the collar or formative epidermal ring surrounding the umbilicus of the feather germ, from which they continue to be derived during regeneration (Pl. II, Fig. 1; and text Figs. 18 and 19).

In descriptions of the development of the feather attention has been directed naturally to the middle layer. The inner layer has been regarded as forming only "accessory structures," and its development has been relatively neglected in recent times, although by no means overlooked by earlier students of feather development (cf. Davies, 1889). At all stages it intervenes between the pulp and the feather. It is very important with reference to the functions of the pulp and therefore requires careful study.

Near the base of the feather germ the stratum cylindricum is composed of a single layer of cubical or cylindrical epidermal cells completely covering the pulp. It clothes the surfaces of each ridge, both centrally, i.e., toward the pulp, and also laterally, i.e., between apposed faces of the ridges (Pl. I, Fig. 5). At the peripheral or outer end of each ridge as seen in cross-section, it extends to the inner layer of the sheath and bends back over the lateral surface of the adjoining ridge.

The stratum cylindricum is thus a single membrane folded in between the ridges; but, with reference to the subsequent development, the two adjacent layers belonging to neighboring ridges will be designated as "barb septa." Each of the latter thus consists of two apposed layers; like the ridges themselves, the barb septa terminate at the rhachis, and they extend between adjacent ridges to their tips. The remainder of the stratum cylindricum, on the other hand, clothes the surface of the pulp directly and completely; it is thus continuous over the surface of the rhachis and, on the opposite side, over the tips of the ridges. It faithfully molds the contours of the ridges and rhachis, so that, when ultimately separated from them after keratinization, it bears their impressions permanently (Pl. III, Figs. 12 and 13).

In an apical direction the part of the stratum cylindricum next to the pulp becomes a stratified epithelium in which two principal layers may be distinguished—an external single layer of squamous epithelium, the "barb membrane," and the remainder next to the pulp, which may be known as the "pulp membrane." Over the apex of the pulp the latter becomes specialized as the "apical membrane of the pulp."

In summary, then, the stratum cylindricum differentiates apically into three parts, viz.: the barb septa, the barb membrane, and the pulp membrane.

C. RESORPTION OF THE PULP

Resorption of the pulp is periodic, and each period of resorption is followed by a period of reconstitution of the pulp and the apical membrane. We distinguish, for purposes of description, three stages of resorption, numbered "I," "II," and "III," and two stages of reconstitution, numbered "IV" and "V." Together, these stages cover the complete cycle of formation of a single pulp cap. Each stage is characterized by typical changes of the apical membrane and of the pulp itself, which are presented in the accompanying diagrams (Fig. 7).

We may start the description with stage V, which represents the completion of reconstitution and immediately precedes stage I of resorption. Three completed caps are represented above the apex of the pulp. It will be understood that the parts shown are completely wrapped within the yet unfolded feather. Three layers are represented in

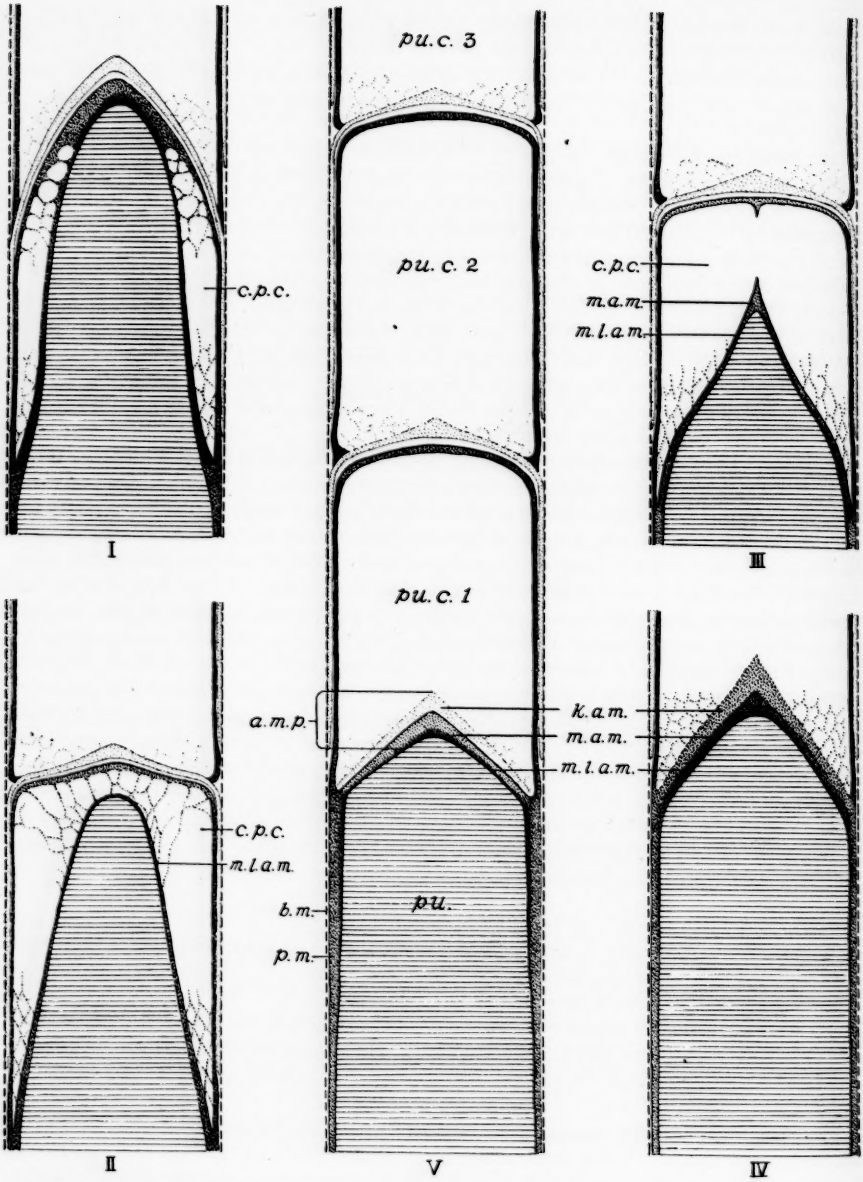

FIG. 7.—Diagrams of the five stages of pulp-cap formation described in text. See table of abbreviations, p. 175.

the ectoderm below the apex of the pulp. The thin external layer represented by a broken line is the barb membrane not concerned in the process of resorption; within this the pulp membrane exhibits two layers which are concerned in periodic processes of resorption, viz.: the Malpighian layer and a thick middle layer.

Over the apex of the pulp it will be noted that the middle layer has formed two layers—an external one, composed of curious keratinized but not flattened cells (cf. Pl. II, Figs. 8, 9, and 10), destined to form the main thickness of the dome of each cap; and an internal one, not further differentiated.

Resorption, of course, concerns the pulp itself; and as it proceeds, the pulp shrinks, first in diameter and then in length, in a manner that can be followed through stages I, II, and III. While this is going on, changes in the ectoderm occur that lead to the production of the cavity of the pulp cap. This cavity arises in the middle layer of the pulp membrane as a large number of vacuoles which gradually run together so as to leave an undivided space. This happens first about the middle of the future pulp cap, and later the cavity extends within the apical membrane itself, so as to separate the pulp completely from the dome of the forming pulp cap (stages II and III).

The pulp is thus left with a thin covering of Malpighian cells and a minimal portion of the middle layer of the pulp membrane. The Malpighian layer then rapidly reconstitutes the apical membrane, as shown in stage IV. In stage V the keratinized layer of the apical membrane is reformed; and, together with its branching extensions, it constitutes the dome of each pulp cap, as shown in Plate III, Figure 13.

The pulp cap, thus formed, has an arched dome consisting of two layers, but the sides include also the barb membrane and are thus three-layered. The diagram greatly exaggerates the thickness of the sides of the pulp cap (cf. Pl. II, Figs. 8, 9, and 10). Subsequently, as the drying-out is completed, the sides become broken off from the dome of the succeeding cap (Pl. III, Fig. 13, *B*), and each cap is then a little thimble of keratin.

These phenomena have not hitherto been described, and they accordingly deserve fuller illustration. We present in Plate II, Figures 8, 9, and 10, photographs of longitudinal sections corresponding to stages I, II, and IV of the diagram. Study of the sections and their captions will serve to correct the account just given, so far as it is diagrammatic.

In the formation of the calamus the same process, periodic even during the final resorption of the pulp, gives rise to a series of "horny" partitions, known of old. The Germans have the curious name "Federseele" for these structures. The term "scala of the calamus" has also been applied to them collectively.

The changes in the pulp that accompany its resorption are conditioned by the highly developed terminal arborization of the axial artery (text Fig. 18; Pl. IV, Figs. 22 and 23). Figures, 8, 9, and 10 of Plate II show the histological pictures for stages I, II, and IV. The apical vessels lose their walls (Pl. II, Figs. 8 and 9), and the blood corpuscles become free in the pulp. These pictures do not, of course, reveal either the sequence or the nature of the events. It is possible that there is a preliminary leucocytosis, but the first action may be autolytic. Presumably the second step is an extensive phagocytosis followed by a restoration of the products of resorption to the blood stream. Reconstitution of the axial artery is to be seen in Plate II, Figure 10 (stage IV), and also diminution of blood corpuscles in the pulp.

In some cases resorption of the axis of the pulp is incomplete—exceptionally in the case of the breast and saddle feathers of the White Leghorn, and quite regularly in the

case of the wing primaries. Under these circumstances a central strand containing haemoglobin and presumable remains of the axial artery runs through the axes of successive caps, binding them together in the manner shown in Plate III, Figure 11.

D. THE PULP CAPS

The pulp caps thus formed are little thimbles of keratin with thickened domes provided with branching processes, as shown in Plate III, Figure 13. The series of caps is at first inclosed by the special barb membrane represented by the broken line in the diagrams (Fig. 7); to this the keratinized barb septa are loosely attached. Figure 12 of Plate III shows the barb septa still in place on the caps. In this figure, *A* is oriented to show the deep impression of the rhachis in the center (the barb septa begin abruptly here in their half-spiral around the cylinder); *B* shows the rhachis groove on the left and exhibits the complete course of some of the septa; and *C* is another view similar to *A*. The septa are lost very quickly as the feather unfolds, but the barb membrane itself

No.	Total Length (Mm.)	Length of Pulp (Mm.)	Length of All Caps (Mm.)	No. of Caps	Average Length of a Cap (Mm.)	Tip Free from Caps (Mm.)
	33 Days' Regeneration					
1	49	12	34	19	1.79	3
2	48	12	32	20	1.60	4
3	50	12	34	22	1.55	4
4	46	13	29	18	1.61	4
5	50	13	33	21	1.57	4
Av.	48.6	12.4	32.4	20	1.62	.....
	37 Days' Regeneration					
6	54	15	38	24	1.60	2

remains adherent to the sides of the caps. Plate III, Figure 13, shows dried pulp caps after loss of the barb ridges.

In the regenerating feathers examined by us, a number of complete pulp caps, varying from one to about five, are usually found beyond the apex of the pulp, wrapped in the barb membrane within the incompletely unfolded barbs. As the unfolding of the barbs is completed, the caps become detached and are lost. It is therefore impossible to determine in the ordinary feather, by count with any considerable degree of accuracy, how many caps are formed in a given period of regeneration of the feather. However, we were fortunate enough to find a number of regenerating saddle feathers of known age in a Brown Leghorn capon in which the sheath of the feather was persistent nearly to the tip (Pl. III, Fig. 14); the feather had therefore not unfolded, and all the pulp caps were retained and could be readily counted.[7] Five of these were plucked on the thirty-

[7] Samuel (1870), in a paper designed for study of the influence of nerves on growth and regeneration of feathers, found that, when the innervation of the wing of the pigeon is severed, the sheath of regenerating feathers persists to the tip, and that they thus appear like long spines. He does not regard this result

third day of regeneration, and the only remaining one with complete sheath was plucked on the thirty-seventh day. The measurements and number of caps in these feathers are given in the table on page 156.

As noted previously, the first cap in the case of saddle feathers of Brown Leghorn capons forms on about the fifteenth day of regeneration. This is subject to some variation, depending on the length of the period of quiescence following plucking and presumably on some other factors; but the amount of variation does not appear to exceed a day usually, or rarely 2 days. In the case of the feathers 33 days of age, the period of cap formation would thus last about 18 days, and at 37 days it would last approximately 22 days. The average number of caps formed in the group of five 33-day feathers is 20, and in the 34-day feathers it is 24, in both cases close to the presumed number of days of cap formation; and the average length of a cap, 1.62 mm., corresponds quite closely to the average daily increment of length of saddle feathers of the same age (cf. Tables 3 and 4).

It was natural, therefore, to suspect at first the existence of a diurnal rhythm. However, in the group of 33-day feathers the number of caps ranges from 18 to 22, which, on such an assumption, would appear to fall a little outside the expected range of variation in time of formation of the first cap. Moreover, if the individual lengths of caps are examined, more variation is found than is consistent with the very regular daily rate of growth of the feather.

If there were a diurnal rhythm, one would expect to find that the various stages of cap formation, and particularly the occurrence of periods of resorption and reconstitution, would tend to be at definite periods of day and night. Accordingly, this possibility, which would be of considerable interest, was carefully examined.

Regenerating breast feathers of from 18 to 25 days of age were plucked at 6:00 A.M., 12:00 M., 6:00 P.M., and 12:00 midnight in consecutive 24-hour periods *on two separate occasions*. Three Brown Leghorn capons were used, and about 12 feathers were taken from each capon at each time, making 288 in all; 277 of them were classified by the stages of cap formation, as shown in the accompanying table.

Time	Resorption			Reconstitution		Total
	Stage I	Stage II	Stage III	Stage IV	Stage V	
6:00 A.M.	10	11	11	17	15	64
12:00 M.	14	14	11	14	25	78
6:00 P.M.	15	12	7	14	19	67
12:00 midnight	7	11	12	20	18	68
Total	46	48	41	65	77	277
Grand total	135			142		277

as due directly to lack of innervation, but rather to the incidental effects of paralysis of the wing. The feathers of the paralyzed wing are never moved or preened. He accordingly attributes the normal loss of the sheath to a point a certain varying distance above the opening of the follicle to movements of the feathers themselves and to preening by the bird, an opinion in which we concur. However, we regard it as probable that other factors, such as abnormal thickness of the sheath, might be responsible for its retention in certain circumstances.

The stages of resorption and reconstitution collectively are approximately equal, and it is therefore probable that they occupy about equal periods of time. Since all stages are found at any hours of the day or night—and this has been confirmed by repeated, less systematic observations—it is necessary to conclude that the periodicity is not determined directly by the regular diurnal fluctuations in metabolic rate (Barott *et al.*, 1938). We must accordingly look for some other causes of the periodicity in this phenomenon.

The problem is obviously related to that of the determination of the length of the pulp, which is quite constant throughout the greater portion of the period of regeneration (Tables 1 and 2; Figs. 2 and 3). The maintenance of an approximate standard of length of pulp specific for different feather tracts indicates some function of length.[8] We have also ascertained that, if apical portions of the pulp are cut off (breast feathers), the remainder tends to regain its original length in the process of further growth. The position that length of pulp has a functional significance is thus reinforced.

We previously postulated a relation between length of pulp and length of barbs (Lillie and Juhn, 1932). It is true that the length of pulp is usually greater than that of the longest barbs; and it may, as a matter of fact, be much longer in certain tracts. But even this does not appear to be invariably true.

The length of pulp bears a regular relationship to the normal length of the sheath, which extends a short distance beyond it, and to the position above the sheath where the barbs unfold and separate from the barb membrane and barb septa. But that this also is not a determining factor is shown by the cases in which the sheath is persistent almost to the tip of the feather, and the pulp caps are nevertheless regularly formed (Pl. III, Fig. 14).

The functional length of the pulp is regulated by the periodic process of cap formation, which involves both a limited amount of resorption of pulp and the accompanying differentiation and reorganization of the apical membrane of the pulp. In ordinary biological terms the function of the apical membrane is obviously to protect the vascular pulp containing 93 per cent of water (see p. 167) from desiccation. The pulp caps above the apical membrane and the thick wrapping of feather and sheath are admirably adapted to reinforce this protection.

It is also obvious, from a biological point of view, that resorption could hardly be a continuous process, like growth. Periodic reorganization of the protective organ after resorption would appear to be necessary in order that fatal exposure should not occur at any step. From the same point of view, admitting that a certain standard of length of pulp has functional significance, resorption cannot be allowed to proceed too far basally at any time, and this distance is actually related to the rate of growth; the most rapidly growing feathers have the longest pulp caps.

The cells of the collar, which is the source of the three layers of the feather germ, are rich in protoplasm, and many of them are in process of division; the collar must therefore contain a large percentage of water. The processes of differentiation progress in an apical direction in all three layers and result in completely desiccated keratinized derivatives. Cell division is found in the bases of the barbs and in the ventral membrane of the shaft for a short distance above the collar; in the case of the sheath, there is no

[8] In the wing primaries the length of the pulp is three to four times greater than in breast or saddle feathers.

cell division above the collar; and in the case of the pulp membrane, multiplication of cells extends to the apex of the pulp. This is evidence that the processes of differentiation and of desiccation proceed at different rates in the three layers of the feather germ; but in all parts there is a gradient in cellular water content, briefly a water gradient, from the base of the feather germ in an apical direction.

The derived structures acquire rigidity according to the degree of keratinization and differentiation, which go hand in hand with loss of water; and it is obvious that, until the degree of rigidity assures independent maintenance of the form of the feather and the interrelations of its parts, it must have outside support and protection against bending and too rapid desiccation. It is probable that adequate rigidity is not attained much below the apex of the pulp, for bending below a certain point results in permanent deformation. While the shaft and barbs of the feather are still soft and pliable, they must be held straight and firmly in position. As the feather develops in the wall of a cylinder, it is necessary that the wall be smooth and of adequate dimensions. The pulp, by its turgor (p. 172), maintains the smooth form of the cylinder against the resistant sheath. It also keeps the surfaces of the barbs moist below the thickening of the apical third of the pulp membrane. The sheath always extends a considerable distance beyond the apex of the pulp, and within this distance the drying of the surfaces of the barbs appears to be completed before they unfold.

This result throws light on the water gradient, the functional length of the pulp, and on the role of the sheath. The functional length of the pulp would appear to be related to the length of the water gradient, and protection against desiccation is strongly emphasized as one of the chief functions of the sheath.

We can thus see clearly that the whole process of cap formation is very perfectly adapted to the requirement of a standard length of pulp, but we do not yet fully understand the nature of this requirement or the physiological mechanisms of the periodicity by which it is regulated.

E. HISTORICAL

There is curiously little information in the literature about the dermal components of the feather germ. Prior to the paper by Davies (1889), which marks a turning-point in our knowledge of development of the feather, the observations are scattered and quite incidental. The pulp was early recognized as the nutritive organ of the feather germ, and it was understood that it underwent complete resorption after the feather was fully formed. The pulp caps were seen even by Malpighi (1697), who derived them from dried-up apical portions of the pulp. Others who referred to the pulp caps were Dutrochet (1819) and Burmeister (1840). The scala in the calamus was early recognized and described. Samuel (1870) made the first experiments on the pulp—indeed, the only ones to date.

Davies (1889), who had a remarkably orderly mind, was the first to state clearly the fundamental principles on which subsequent interpretation has rested. The pulp grows from its base and is resorbed at its apex;[9] neither the process of resorption nor its rate was described in detail, but he connected the formation of pulp caps with the process of resorption and derived them correctly from the stratum cylindricum. He did not, however, observe the apical membrane of the pulp, and postulated, instead, periodic contractions and concomitant thickening of the stratum cylindricum over the apex of

[9] Hosker (1936, p. 164) erroneously ascribes to Davies her own mistaken view that the pulp is resorbed at the base.

the resorbing pulp. He also implied that the scala in the calamus is similarly formed. He derived the barb septa also from the stratum cylindricum.

The subject has rested in about the form that Davies gave it down to the present time. Hosker (1936), in her very comprehensive paper, devotes some attention to the pulp and the pulp caps. She states:

> As the pulp passes down the feather, the cylinder cells become congested, and the outermost ones, being further from the nourishment contained in the abundant blood supply of the pulp, are overtaken by the process of cornification. This occurs at the sides as well as the tip of the pulp, fig. 37, Plate 22, so that a series of cones is formed, connected with each other.
>
> In this way the feather caps arise, and, where protected by the calamus, persist, although distal to the superior umbilicus they soon break away from the feather.[10]

The figure referred to is a drawing quite similar to our photograph (Pl. II, Fig. 9), but it is misinterpreted, owing to her mistaken opinion that the pulp is resorbed at the base.

4. EARLY HISTORY OF THE PULP

The measurements of the relative lengths of pulp and feather in breast and saddle recorded in Tables 1–3 and in the curves in Figures 2 and 3 begin on the thirteenth and fourteenth days of regeneration. At this time the regenerating feather has emerged from the mouth of the follicle, and the tip of the originally closed sheath has been lost, so that a tuft of barbs extends beyond the sheath. Before this time the sheath is closed over the tip of the feather; hence the common term "pinfeather."

Growth during the pinfeather stages exhibits certain special features. It is decidedly slower during about the first 9 days, while still concealed in the follicle, than later (Lillie and Juhn, 1932). This period is concerned with the primordial organization of the feather germ, during which the elements of the tip of the feather are laid down.

During the same period the sheath and the pulp are much longer than the feather proper (Fig. 15); the stratum intermedium is absent over the upper portion of the pulp; and the stratum corneum (sheath) and the stratum cylindricum (pulp membrane) are in immediate contact here. The sheath is thick and strongly keratinized, and the pulp membrane has developed a special apical membrane over the tip of the pulp.

At about the eighth day an apical cavity (Fig. 15, *a. c.*) begins to appear between the tip of the pulp and the sheath, which rapidly increases in length until the loss of the closed apex of the sheath, when it opens to the exterior between the tuft of apical barbs. Measurements show that the distance from the dorsal barbs to the tip of the sheath (Fig. 15, 1 + 2) remains quite constant until the loss of the sheath, about the thirteenth day; during this time the total length of the feather germ (Fig. 15, *1*) has increased from approximately 3 mm. (eighth day) to 9.5 mm. That is to say, the feather has grown during this period, as also later, at the same rate as the sheath. On the other hand, the distance between the barbs and the apex of the pulp (Fig. 15, 2) diminishes steadily during the same period, and the barbs come to exceed the pulp in length. Simultaneously, the length of the apical cavity (Fig. 15, 1) increases *pari passu*. The latter facts are to be explained by resorption of the pulp at its apex,[11] which begins about the eighth day, resulting in the formation of the apical cavity. Between the eighth and

[10] Hosker, 1936, p. 163.

[11] The typical pattern of circulation with special provision for resorption of the apex of the pulp has developed by, or before, the ninth day of regeneration (cf. p. 173).

the thirteenth days, however, the rate of growth of the pulp exceeds the rate of resorption, so that a net increase in length results until the functional length of the pulp is attained (cf. Figs. 2 and 3). We therefore reach the conclusion that, after the feather germ is once organized, the pulp and the ectodermal components grow at the same axial rate, as we have shown to be the case in later stages.

Resorption of the pulp is periodic during the pinfeather stages. As in later stages, the apical membrane exhibits successive periods of keratinization of its outer cells, followed by splitting-off. To this extent the process resembles the formation of pulp caps in later stages; but the partitions across the apical cavity thus resulting, corresponding to the domes of the typical pulp caps, are relatively very delicate, and proper pulp caps are not formed.

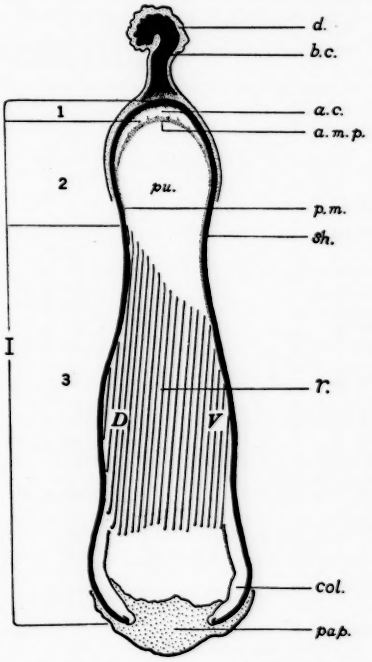

FIG. 15.—Feather germ of 8 days' regeneration from the breast, Brown Leghorn capon, dissected out of the follicle. Entire preparation from the right side; the dorsal surface (*D*) is to the left and the ventral surface (*V*) to the right. The tip of the preparation bears a blood coagulum formed as a result of plucking, inclosed within debris from the wall of the follicle. *I* = total length of feather germ; 1 = extent of the apical cavity; 2 = length from the dorsalmost barb to the tip of the pulp; 1+2 = distance from the dorsalmost barb to the apex of the sheath; 3 = distance from the umbilicus to the tip of the dorsalmost barb. × *ca.* 25.

5. THE WEIGHT OF THE PULP COMPARED WITH THE FEATHER

The preceding section deals with the linear dimensions of the pulp. The present section presents our data on weight of pulp produced.

In order to determine weights, it is necessary to pluck the feather, and this precludes the possibility of using the same feather for different ages of regeneration. The age comparisons are, therefore, those of different feathers, and this introduces sources of error that it is impossible to eliminate entirely. We have sought to reduce them as much as possible in the following ways: (1) by using the same bird for a complete set of determinations and keeping the records for different birds separate, (2) by using only feathers of known location and regeneration age, and (3) by averaging for each stated figure the results on 5 neighboring feathers of the same bird (in the case of saddle feathers).

In spite of the difficulties, consistent results have been obtained. This comes out strikingly in the comparison of weights of constituents in the saddle feathers of two capons given beyond, which give practically identical results. The weights determined are those of the entire plucked feather; of the feather after removing the pulp; and of the pulp itself, calculated as the difference between the first two (Tables 5 and 6). The data of Table 5 are plotted in the graphs of Figure 16.

A. SADDLE FEATHERS (CAPONS 171 AND 148)

Practically the entire saddle region was plucked simultaneously in each bird on December 28, 1938. Beginning on the fourteenth day of regeneration (capon 171) or the thirteenth day (capon 148), the weights given in Tables 5 and 6 were determined on

TABLE 5

BROWN LEGHORN CAPON 171: SADDLE FEATHER*

Age (Days)	Total Weight (Cgm.)	Weight of Feather (Cgm.)	Weight of Pulp (Cgm.)†	Percentage of Pulp Weight to Total Weight
14	17.0	8.0	9.0	52.9
16	26.0	13.0	13.0	50.0
18	34.0	16.0	18.0	52.9
20	40.0	19.5	20.5	51.2
22	46.0	22.7	23.3	50.7
24	49.5	25.5	24.0	48.5
26	53.0	28.2	24.8	46.8
28	56.0	30.2	25.8	46.1
30	58.2	32.5	25.7	44.2
32	60.0	34.5	25.5	42.5
33	60.7	35.4	25.3	41.7
35	62.0	37.0	25.0	40.3
37	63.5	38.5	25.0	39.4
39	64.3	40.0	24.3	37.8
41	65.0	41.2	23.8	36.6
43	65.7	42.2	23.5	35.8
45	66.4	43.0	23.4	35.4
47	67.0	43.3	23.6	35.2
49	67.5	44.0	23.5	34.8
51	67.7	44.5	23.2	34.4
53	68.0	45.0	23.0	33.8
55	68.2	45.5	22.7	33.3
57	68.3	46.2	22.1	32.4
59	68.4	46.7	21.7	31.7
61	68.3	47.5	20.8	30.5
63	68.3	48.0	20.3	29.7
65	68.1	49.0	19.1	28.0
67	68.0	50.0	18.0	26.5
69	67.7	51.0	16.7	24.7
71	67.5	52.5	15.0	22.2
73	67.0	54.0	13.0	19.4
75	65.5	55.0	10.5	16.0
77	63.6	55.7	7.9	12.4
79	61.5	56.7	4.8	7.8
81	58.7	55.7	3.0	5.1
83	56.0	55.0	1.0	1.8
85	54.0	54.0	0.0	0.0

* Weight of pulp in relation to total weight of feather. Total weight of 5 feathers, multiplied by 2, entered in centigrams. The same feathers which were used for length determinations in Table 3 were used here.

† The sum of all determinations of weight of pulp, amounting to 690 cgm., is referred to on p. 165.

alternate days in each case to the completion of growth of the feathers. (Note that after the thirty-second day the weighings were made on the same day for both birds.) Five closely placed regenerating feathers were plucked each time between 8:00 and 9:00 A.M. and weighed together; this weight, in centigrams, multiplied by 2, is entered as total weight, which is therefore ten times the average weight of the 5 feathers. No atten-

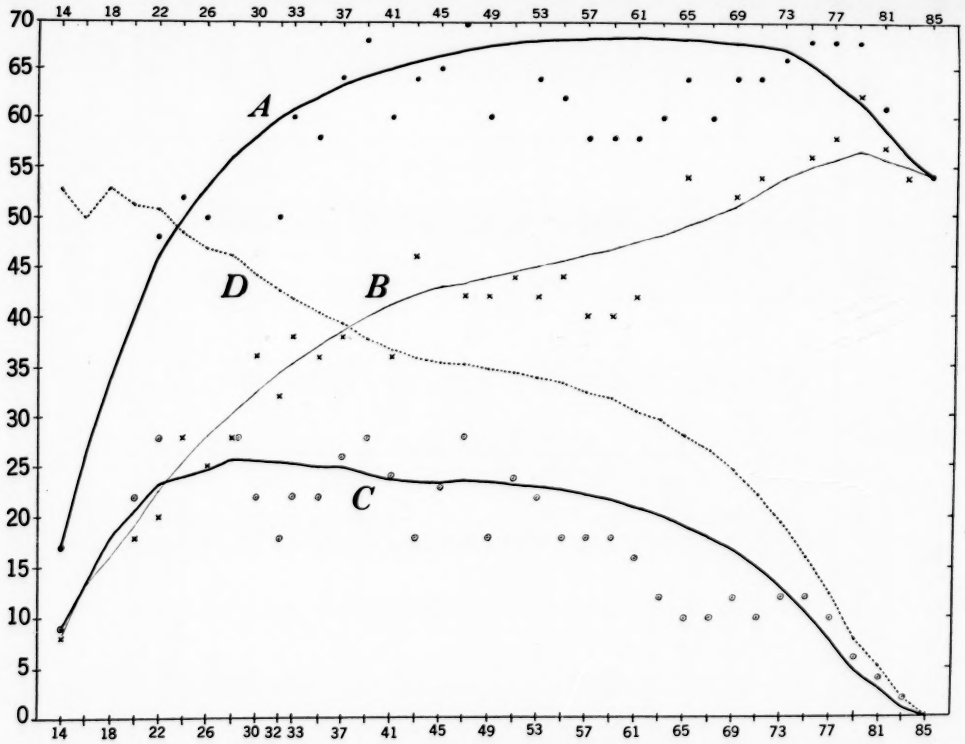

FIG. 16.—Curve of weights of constituents of regenerating saddle feathers, Brown Leghorn capon 171. Data from Table 5. *A* = total weight; *B* = weight of feather; *C* = weight of pulp; *D* = percentage of weight of pulp to total weight. Abscissae = days of regeneration; ordinates = centigrams for curves *A*, *B*, and *C*, percentages for curve *D*. Weight is ten times the weight of a single feather.

tion was given to position in the entire field of regenerating feathers in successive pluckings, and this may be one of the sources of irregularity in the weight curves.

The pulp was then carefully removed, and the 5 feathers were weighed together again. This weight, multiplied by 2, is the weight of the "feather" given in the third

TABLE 6

BROWN LEGHORN CAPON 148: SADDLE FEATHER*

Age (Days)	Total Weight (Cgm.)	Weight of Feather (Cgm.)	Weight of Pulp (Cgm.)†	Percentage of Pulp Weight to Total Weight
13	16.0	10.0	6.0	37.5
15	26.0	13.0	13.0	50.0
17	35.0	16.5	18.5	52.9
19	42.5	20.0	22.5	52.9
21	48.0	22.5	25.5	53.1
23	52.5	25.0	27.5	52.4
25	55.0	27.0	28.0	50.9
27	58.0	29.0	29.0	50.0
29	60.0	30.0	30.0	50.0
31	62.0	31.2	30.8	49.7
33	63.5	32.5	31.0	48.8
35	65.0	34.0	31.0	47.7
37	66.0	35.0	31.0	47.0
39	67.5	36.0	31.5	46.7
41	68.2	37.5	30.7	45.0
43	68.8	39.0	29.8	43.3
45	69.1	40.0	29.1	42.1
47	69.5	41.5	28.0	40.3
49	69.6	42.5	27.1	38.9
51	70.0	43.5	26.5	37.9
53	70.0	44.5	25.5	36.4
55	69.8	45.5	24.3	34.8
57	69.6	46.5	23.1	33.2
59	69.5	47.0	22.5	32.4
61	69.0	47.5	21.5	31.2
63	68.5	48.0	20.5	30.0
65	68.0	48.5	19.5	28.7
67	67.5	49.5	18.0	26.7
69	66.5	50.0	16.5	24.8
71	65.5	51.0	14.5	22.1
73	64.0	51.7	12.3	19.2
75	61.5	52.5	9.0	14.6
77	59.5	51.7	7.8	13.1
79	56.0	51.0	5.0	9.0
81	53.0	50.5	3.5	6.6
83	50.0	50.0	0.0	0.0

* Weight of pulp in relation to total weight of feather. Total weight of 5 feathers, multiplied by 2, entered in centigrams. The same feathers which were used for length determinations in Table 4 were used here.

† The sum of all determinations of weight of pulp, amounting to 670 cgm., is referred to on p. 165.

column. The weight of the pulp is then entered as the difference between the total weight and the weight of feathers, and the percentage weight of the pulp is calculated. These feathers are the same ones that were used for determination of the total length and the length of pulp given in Tables 3 and 4. In capon 171 there were 185 feathers used in all, and 180 in capon 148.

From the figures of length and weight given in Tables 3 and 5 (capon 171) and in Tables 4 and 6 (capon 148) the following calculations may readily be made:

1. The average weight of 1 mm. of pulp during the entire period of regeneration is 69 cgm. ÷ 443.3 mm. = 0.155 cgm. in the case of capon 171, and 67 cgm. ÷ 430.6 mm. = 0.155 in the case of capon 148; thus the same in both.

2. The total weight of pulp produced for each feather during regeneration is 129 × 0.155 = 20 cgm. in the case of capon 171, and 119.5 × 0.155 = 18.5 cgm. in the case of capon 148.

TABLE 7

WHITE LEGHORN COCK: ANTERIOR BREAST FEATHER*

Age (Days)	Total Weight (Cgm.)	Weight of Ectodermal Component (Cgm.)	Weight of Pulp (Cgm.)†	Percentage of Pulp Weight to Total Weight
13	27.4	13.9	13.5	49.3
14	28.0	14.0	14.0	50.0
16	31.2	15.2	16.0	51.3
18	35.5	17.5	18.0	50.7
20	40.0	20.0	20.0	50.0
22	43.3	22.5	20.8	48.0
24	46.0	25.0	21.0	45.7
26	48.0	27.0	21.0	43.8
28	50.8	30.0	20.8	40.9
30	53.5	33.0	20.5	38.3
32	55.8	35.5	20.3	36.4
34	58.0	38.0	20.0	34.5
36	61.9	42.5	19.4	31.3
38	63.1	45.0	18.1	28.7
40	62.2	47.0	15.2	24.4
42	60.5	48.5	12.0	19.8
44	54.	46.2	8.0	14.8
46	48.5	44.5	4.0	8.3
48	44.8	42.5	2.3	5.1
50	42.4	41.0	1.4	3.3
52	42.1	41.0	1.1	2.6
54	41.0	41.0	0.0	0.0

* Weight of pulp in relation to total weight of feather recorded for a single feather in each entry; each entry selected to correspond as closely as possible to the feathers measured for length in Table 1.

† The sum of all determinations of weight of pulp, amounting to 307.4 cgm., is referred to on p. 166.

3. The weight of 1 feather produced during regeneration in the case of capon 171 is 5.4 cgm., and in the case of capon 148 is 5 cgm.

4. Capon 171 requires 20 ÷ 5.4 = 3.7 cgm. of pulp to produce 1 cgm. of feather, and capon 148 requires 18.5 ÷ 5 = 3.7 cgm. to produce 1 cgm. of feather.

B. BREAST FEATHERS

The same method used for the saddle feathers was used also for the breast feathers to determine a curve of weights, as well as lengths of pulp and of feather during regeneration. However, the range of size of feather is so considerable in the breast tracts that, in using as many feathers as are necessary, a much higher degree of irregularity of the

curves than in the saddle tract was unavoidable. Accordingly, these figures were discarded and another method used.

Growth in length was measured *in vivo* in a White Leghorn cock for each of a group of 3 feathers as described on page 145; and a fourth feather from the same region, agreeing with the average of these three in age and measurements, was plucked and used for determination of weights and other purposes previously mentioned. The figures of weight were then multiplied ten times and are so entered in Tables 7 and 8. This

TABLE 8

WHITE LEGHORN COCK: POSTERIOR BREAST FEATHER*

Age (Days)	Total Weight (Cgm.)	Weight of Feather (Cgm.)	Weight of Pulp (Cgm.)	Percentage of Pulp Weight to Total Weight
13	27.5	13.5	14.0	50.9
14	32.0	15.5	16.5	51.6
16	39.5	20.0	19.5	49.4
18	45.7	24.5	21.2	46.4
20	52.5	28.5	24.0	45.7
22	60.0	32.5	27.5	45.8
24	65.0	36.0	29.0	44.6
26	69.5	40.0	29.5	42.4
28	73.5	43.0	30.5	41.5
30	77.0	46.5	30.5	39.6
32	79.5	50.0	29.5	37.1
34	81.5	53.0	28.5	35.0
36	82.5	55.0	27.5	33.3
38	83.2	57.5	25.7	30.9
40	83.5	59.7	23.8	28.5
42	83.5	61.7	21.8	26.1
44	83.5	63.5	20.0	24.0
46	83.2	65.0	18.2	21.9
48	83.0	66.0	17.0	20.5
50	82.5	67.5	15.0	18.2
52	81.3	68.5	12.8	15.7
54	79.7	69.5	10.2	12.8
56	77.2	69.0	8.2	10.6
58	73.5	68.0	5.5	7.5
60	70.0	66.5	3.5	5.0
62	65.0	65.0	0.0	0.0

* Weight of pulp in relation to total weight of feather recorded for a single feather; each entry selected to correspond as closely as possible to the feathers measured for length in Table 2.

method has the disadvantage of using only a single feather for each determination; but, on the other hand, the advantage of control by known feathers.

This was done separately in the anterior part and the posterior part of the breast tract, and the two independent results are a check on each other. The measurements are given in Tables 7 and 8, and the data of Table 8 are entered in graphs (Fig. 17).

The same calculations may now be made as in the case of the saddle feathers:

Anterior breast.—

1. The average weight of 1 mm. of pulp during the entire period of regeneration is $30.74 \div 215.2 = 0.143$ cgm.

2. The total weight of pulp produced for each feather during regeneration is 79 × 0.143 = 11.297 cgm.

3. The total weight of 1 feather produced during regeneration is 4.1 cgm.

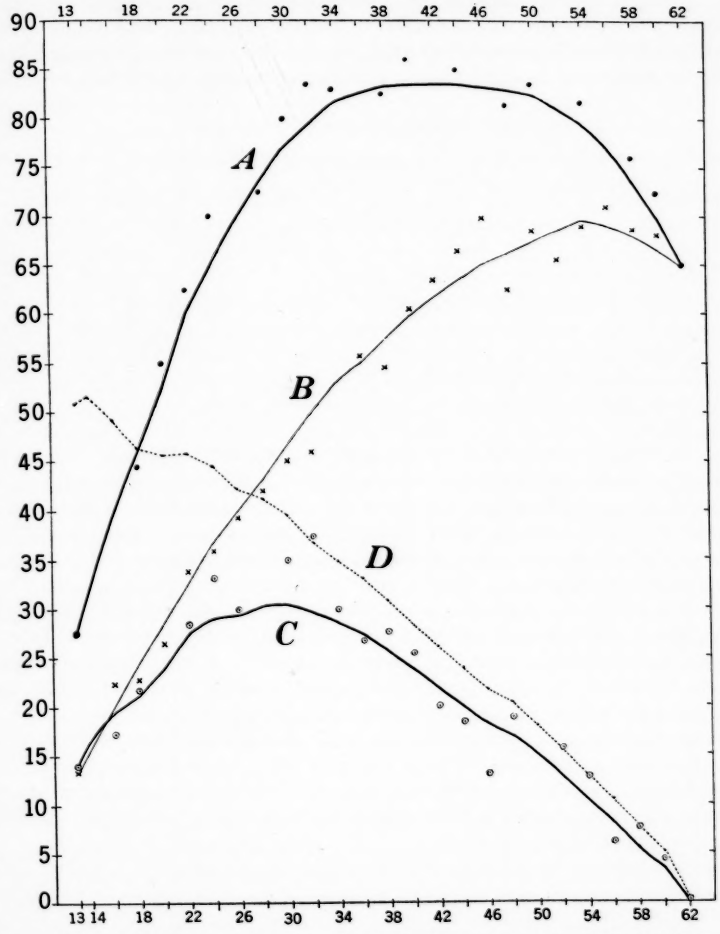

FIG. 17.—Curve of weights of constituents of regenerating posterior breast feathers, White Leghorn cock. Data from Table 8. *A* = total weight; *B* = weight of feather; *C* = weight of pulp; *D* = percentage of weight of pulp to total weight. Abscissae = days of regeneration; ordinates = centigrams for curves *A*, *B*, and *C*, percentages for curve *D*. Weight is ten times the weight of a single feather.

4. Pulp weighing 11.3 cgm. has produced 4.1 cgm. of feather, a ratio of 2.76:1.[12]

In order to gain an idea of the quantity of pulp required for a complete molt, let us

[12] In order to provide an independent check on the ratio between weights of pulp and feather, 108 regenerating feathers from the breast of a Brown Leghorn were plucked on the eighteenth and nineteenth

take a ratio of 3:1 as about the average ratio of weights of pulp and feathers.[13] All the feathers of a young White Leghorn cock, weighing 1,642 gm., were found to weigh, after drying, 105 gm.; the pulp requirement for a complete molt in the case of this bird would thus be 315 gm. The dry weight of the feathers plus pulp, 420 gm., is 25.5 per cent of the weight of the entire bird. This gives a rough idea of the quantitative strain of molting, quite apart from the specific chemical requirements of the metabolism involved. According to our actual determination, the pulp contains 93.65 per cent of water.

IV. THE BLOOD VESSELS OF THE PULP[14]

1. INTRODUCTION

The blood vessels of the pulp were studied by injection with gelatin carmine for the most part; but for confirmation of certain points, sections of uninjected feather germs were also used. We have not yet succeeded in studying the circulation in the pulp *in vivo*. Areas with regenerating feathers of known, or in some cases unknown, age were selected; the skin was reflected with the bird under anesthesia, and a small artery, or in some cases a vein, was injected peripherally through a fine hypodermic needle with the warmed mass. In this way a considerable number of feather pulps could be injected simultaneously. The mass sets rapidly. The injected area was then excised, hardened in formalin, and the feathers prepared individually for study.

After some experimenting with the Brown Leghorn, the White Leghorn was used exclusively in order to get rid of the obscuring effects of melanophores. The method of study most frequently used was to dissect the feather and follicle together from the skin and to bisect the pulp-containing portion with a sharp razor blade; both halves were then cleared and mounted together in balsam. Figures 18 and 19 were made from such preparations with aid from a series of stained longitudinal sections.

In other cases the follicle was opened and the injected pulp was dissected free from the sheath and barbs; the bottom of the follicle might be left attached, but reflected, to show relations of the blood vessels of the follicle[15] to excurrent vessels of the pulp; other anatomical dissections were also made. Some entire pieces of injected skin, with the feathers cut off short, were cleared and mounted whole to show the relations between the external cutaneous vessels and the blood vessels of the pulp.

In such ways many preparations of breast, neck, and wing feathers of different stages of regeneration were made, of which over 150 have been retained as permanent preparations. The following account is based mostly on the breast feathers in order to simplify the matter as much as possible; but there seem to be no differences in principle between the feathers of the different tracts.

The blood circulation in the pulp is quite unique in certain of its features, owing to special functional requirements.

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days, and the pulp and feather separated. The pulps were weighed in groups of five, and each feather was weighed separately. From these data the average weights per millimeter of pulp and feather were calculated to be 0.172 cgm. and 0.0516 cgm., respectively—a ratio of 3.33:1.0.

[13] No account has been taken in these calculations of the amount of the sheath lost during regeneration.

[14] There are no previous accounts of the circulation in the pulp. Peska (1927) described the first origin of the vascular loop in the embryonic feather papilla.

[15] It may be noted incidentally that the walls of the follicle have an extremely rich and characteristically arranged blood supply.

1. Growth of the feather is so rapid that a very rich blood supply is necessary, especially in the basal region adjacent to the collar, where all the plasmatic growth occurs; an especially rich capillary network occurs here.

2. The need for constant resorption and removal of the apex of the pulp at the same rate that it grows basally requires a very extensive apical capillary network; the pulp membrane and apical membrane also need nutrition for their extensive and rapid periods of reconstitution. Hence the maintenance of a large arterial supply and excurrent system to and from the apex of the pulp.

3. Another important requisite to be fulfilled in the blood system of the pulp depends on the fact that it is constantly growing out with the pulp at a rate of 2–3 mm. per day or even faster. It follows that the vascular system, like the epidermal system and the pulp itself, is constantly regenerating at its base at the same rate as they. Hence, in order to preserve a rather constant pattern throughout the greater part of the period of regeneration, as it does, it is necessary that it be extremely labile. Only the arteries possess adventitious coats, and the entire system within the pulp, whether in the form of capillaries or large sinuses, is otherwise exclusively endothelial. It is only within the papilla that the excurrent vessels acquire adventitious coats and may properly be denominated "veins."

4. The relatively narrow portals of entry and exit of the blood (Figs. 18 and 19) suggest a mechanism for retention of a large amount of blood constantly within the pulp.

2. THE MORPHOLOGY OF THE VASCULAR SYSTEM OF THE PULP

After these preliminaries we may proceed next to describe the actual vascular pattern of a given stage of the breast feather. Each regenerating feather is supplied by a single axial artery, derived from a cutaneous artery (Fig. 18), which traverses the entire length of the pulp and ends in an apical arborization. The part of the axial artery within the papilla is very thick walled (cf. Pl. II, Fig. 1). Its lumen through the papilla is extremely narrow in the preparations, although usually with a single knotlike swelling. At the apex of the papilla the lumen enlarges suddenly into the axial artery of the pulp, which is relatively very thin walled, although it and the main branches possess adventitious coats.

The branches of the axial artery as shown in Figure 18 are based on a single preparation. It will be noted that those at the base are relatively small, which, at first sight, appears incongruous in view of the vascular needs of the corresponding portion of the feather germ; but, as explained before, the entire system is moving out with the growth of the pulp, and the branches toward the base are the newer ones.

Photographs of the apical arborization of the axial artery are shown in Plate IV, Figures 22 and 23. Figure 22 represents the arborization at about stage II of the resorption of the pulp, and Figure 23 at about stage IV.

The blood in the branches of the axial artery passes into capillaries, which reunite to form the excurrent system, as shown in Figure 19, the pattern of which is likewise based on a single preparation. All the capillaries and excurrent vessels have only endothelial walls, no adventitious coats whatever being present.

The outstanding features of the excurrent system[16] (Fig. 19) are: (1) The series of large pericentral sinuses which occur invariably in the basal portion of the pulp and

[16] As the excurrent vessels within the pulp are entirely devoid of adventitious coats, these vessels are not denominated "veins."

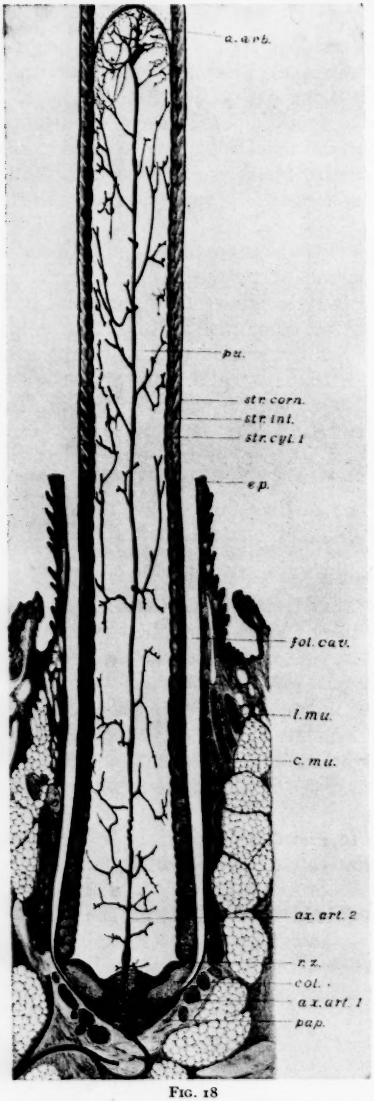

FIG. 18

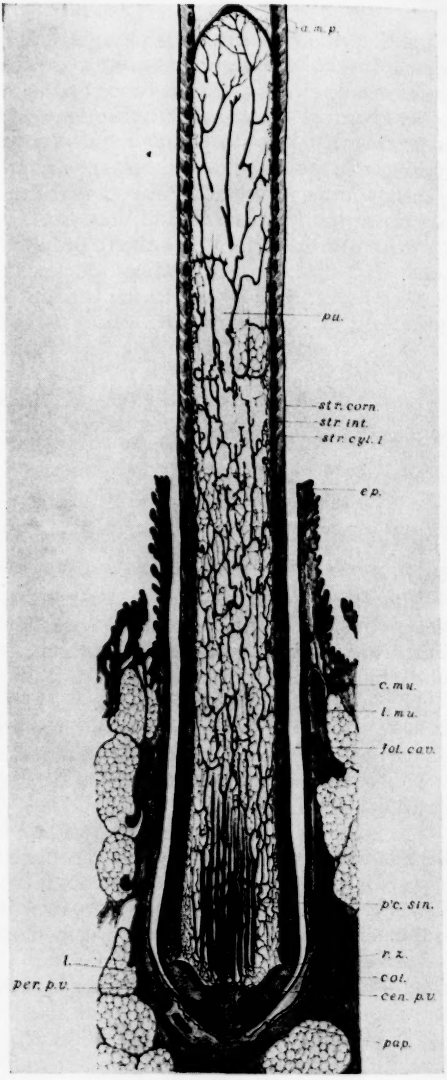

FIG. 19

FIG. 18.—Semi-diagrammatic representation of the arterial circulation in the pulp and papilla of a breast feather on the twenty-first day of regeneration, White Leghorn cock. ×18. By Hsi Wang. See table of abbreviations, p. 175.

FIG. 19.—Semi-diagrammatic representation of the capillary and excurrent system of the pulp and papilla of a breast feather on the twenty-first day of regeneration, White Leghorn cock. ×18. By Hsi Wang. See table of abbreviations, p. 175.

occupy about one-seventh of its entire length. The photographs shown in Plate II, Figure 1, and Plate I, Figure 4 (cf. also Pl. IV, Figs. 20 and 21), give an excellent idea of the disposition of the sinuses. (2) The extremely narrow true veins within the papilla arising from the looped connections of the pericentral sinuses. These veins are irregular in number; some of them occupy the central region of the papilla and hence are called "central papillary veins"; others, "peripheral papillary veins," are found on the periphery of the papilla. It will be seen (text Fig. 19 and Pl. IV, Fig. 21) that they communicate externally with sinuous cutaneous veins. (3) The very richly developed capillary network at the periphery of the pulp in the region occupied by the sinuses, which we regard as related to the very active growth in this region.

Figures 20 and 21 of Plate IV are photographs of longitudinally bisected feather germs, including the region of the papilla and part of the region of the sinuses.

3. DISCUSSION

The general morphology of the system is related, as suggested in the introduction to this section, to the functional requirements of metabolism, resorption, and maintenance of a constant rich supply of blood in the pulp. In considering these relations, we should discuss the course of the blood stream, the pattern in different stages, the lability and regulation of the pattern with reference to the continuous outward growth of the pulp, and the special features of pattern in different tracts.

As stated before, we have not succeeded in observing the course of the blood stream within the pulp *in vivo*. Dr. Melvin Knisely was so kind as to aid us in special illumination of the pulp-containing part of the growing feathers of young White Leghorn chicks, but it was found to be impossible to obtain any clear views through the sheath and ridges of the germ. Exposure of the pulp in such a way as to preserve normal circulation was also unsuccessful.

We have to depend, therefore, upon the anatomical relations exclusively for conclusions concerning the course of the blood within the pulp. The general course is unmistakable. The axial artery and its branches distribute the blood at various levels, in the manner shown in Figure 18, to the capillary network which is predominantly peripheral. These capillaries reunite in larger, more central vessels, which, like the capillaries, have only endothelial walls; and these lead into the large basal pericentral sinuses, also with exclusively endothelial walls, which discharge through the central and peripheral papillary veins in the manner already described.

The axial artery and the veins within the papilla possess very narrow lumens relative to their connections within the pulp. It would be expected that the process of fixation necessary to the preservation of specimens for study would tend to contract the walls, and hence it is not safe to conclude that the condition found in fixed specimens is typical of their condition under all functional conditions. The walls of the axial artery are very thick (cf. Pl. II, Fig. 1); the appearance of an enlargement of the lumen at the base of the papilla, as shown in Figure 18, is very common. It is quite probable that the lumen of the artery throughout its extent is capable of dilation and contraction, and thus of regulating the amount of blood admitted to the pulp.

The capacity of the papillary veins collectively is greater than that of the single axial artery. In some preparations they appear greatly distended. Possibly the muscular substance of the papilla controls this to some extent.

It is not certain that all of the blood passes through the sinuses or that all of the

blood in the sinuses is on the direct way out. The cross-connections between the sinuses and the peripheral capillaries may be two-way channels responsible in part for the metabolic requirements of this very active portion of the feather germ; the arterial system is, in fact, very poorly developed in this region, owing to its constant renewal as the pulp grows out from the papilla.

The narrowness of the portals of entry and exit of blood with relation to the capacity of the vessels of the pulp can best be interpreted as a device to store and conserve within the pulp quantities of blood adequate for the great demands of growth and resorption. It is quite probable that the substance of the papilla acts as a regulator of the amount of blood within the pulp. The sinuses might then be interpreted as reservoirs to meet the demands of the growing region of the germ so that this would not be subject to sudden fluctuations. The quantitative demands for blood during replacements after molting or extensive plucking are great and undoubtedly also involve changes in the general cutaneous blood system; but the highly localized requirements of the individual feather can best be met by local adaptations.

We have also considered the question whether the wall of the follicle might function by contraction in regulating the amount of blood in the pulp. The strong layer of circular muscles in the wall next to the lining epidermis (Figs. 18 and 19) suggests this possibility. Experiments by Kuhn (1932) and Woitkewitsch (1934*a*, 1934*b*) agree in showing that the follicle plays no necessary role in regeneration of the feather. We have also confirmed this conclusion. The follicle may be opened from top to bottom, so as to prevent any compression by it on the growing feather without hindering regeneration. Woitkewitsch even diverted the growing feather outside its follicle through a small opening made at its base, with the result of a mere retardation of rate of growth as consequence. We conclude, therefore, that the wall of the follicle cannot play an important role in regulating the blood supply within the pulp.

A final suggestion on regulation of the blood supply within the pulp concerns the question of turgor within the pulp itself. Although the pericentral sinuses may be full of blood, practically none of it escapes when the regenerating feather is plucked. But if the base of a freshly plucked regenerating feather is gently pressed, a clear serum[17] exudes, along with a smaller amount of blood. The question is thus suggested whether turgor of the pulp is maintained by the serum and may be a factor in regulating the amount of blood within the pulp.

That turgor of the pulp is not sustained by direct blood pressure appears to be proved by the fact that even considerable loss of blood after amputation of apical fractions of the pulp causes only a brief halting of the regeneration of the feather (unpublished observations). The plain inference of this fact is that the relation between the pulp and the ectodermal cylinder has not been seriously disturbed, and that shrinkage of the pulp due to loss of blood does not occur. There is thus only an indirect relation between the blood and the turgor of the pulp. But the relationship is, nevertheless, very immediate, for if the blood supply of the regenerating feather is cut off by ligaturing the axial artery, the pulp does not swell when the sheath is removed, as it does immediately if the circulation be left intact (see p. 144); this demonstrates that turgor of the pulp is lost very rapidly after the circulation is shut off, though persisting after bleeding

[17] A similar clear serum also exudes from the tip of the pulp when the apical membrane is removed. It is evident that, in addition to extracellular supporting substance, there is a fluid extravascular component of the pulp.

at the apex. The indications are that the pulp is constantly extracting fluid from the blood stream and that it is this fluid which is responsible directly for the turgor of the pulp.

The functional requirements of the circulation at different stages are essentially the same up to the time when the definitive diminution of the pulp begins (Figs. 2 and 3). Correspondingly, the general pattern of vascularization is the same throughout the entire plateau period of length of the pulp. This is the case in spite of the fact that the entire system is constantly being carried out by the basal growth of the pulp and new vascular elements are being formed in the new growth. Even at 9 days of regeneration in the breast feathers of White Leghorns, one finds the same narrow portals of entry and exit, the axial artery with its apical arborization, and the large basal pericentral sinuses. During the period of definitive diminution of the pulp, as shown in Figures 2 and 3, the principal change appears to be in the reduction of the pericentral sinuses.

The constancy of general pattern with reference to fixed levels from the base of the germ, in spite of continuous outward growth with reference to these levels, demonstrates a high degree of capacity for regulation of the system. This is based on the fact that, with the exception of the arterial system, the entire complex is endothelial and is embedded in a loose and pliable mesenchyme. The system is therefore free to expand or to contract with reference to the pressure and volume of the blood flow. This, in its turn, bears an obvious relation to the state of functional activity at various levels, which must be supposed to control vascular reactions according to well-known biological principles.

The pulp, growing constantly from the surface of the papilla, is supplied with capillaries as rapidly as it is formed, and this does not seem to involve any adaptations special to the particular system. It is different, however, in the case of the axial artery, which requires adaptations special to the moving system of which it forms a part. In the first place, its base must be continuously renewed without detriment to the circulation; and, in the second place, the new portion of the pulp must be supplied from time to time with new arteries.

The part of the axial artery immediately above the apex of the papilla exhibits special features correlated with these requirements. It is much dilated, as compared with the portion within the papilla; and there is a sudden transition from the thick wall of this portion to the much thinner wall within the pulp (Pl. II, Fig. 1). The dilation above the papilla is often considerably reduced apically, which would seem to indicate a readier yielding to the internal blood pressure at this place. Minute arteries bud out of the dilation. Although these buds end blindly in the pulp at first, they soon connect with the rich capillary system; and, as they are carried apically, they enlarge. Thus, at any given time the basal branches of the axial arteries are the smallest ones, and there is a progressive increase in size apically, for a certain distance at least (see Fig. 18).

The circulation in the neck hackles and in the wing primaries has also been studied in numerous injected specimens for comparison with breast feathers; other tracts were not studied. The general pattern of the circulation is the same as in the breast feathers. Of course, numerous differences in detail appear. In the wing primaries there is a great increase of scale in diameter and especially in length, with a corresponding increase in the capacity of the main vessels. The sinuses appear to be more numerous, and the central veins of the papilla decidedly larger, whether relatively so or not. But it is clear

that there is a type of circulation in the pulp of regenerating feathers to which all those studied conform.

V. SUMMARY

The growth of the pulp of regenerating breast and saddle feathers of White and Brown Leghorn fowl has been examined with reference to the growth of the feather proper. The pulp grows in length at the same rate and to the same extent as the feather. However, after about the fifteenth day of regeneration periodic resorption of the apex of the pulp equals the growth, which proceeds from the base, so that a certain standard, or functional, length of pulp is maintained throughout the greater part of the period of regeneration until about the beginning of formation of the calamus, after which growth slackens and the pulp is finally completely resorbed in periodic steps marked by the formation of the scala of the calamus.

The periods of resorption of the pulp of about 12 hours' duration are approximately daily, though not conforming to any fixed hours of day or night. Periods of reconstitution of about equal duration alternate with them. The periods of resorption are marked by a profuse phagocytosis in the pulp and by splitting of the thickened ectodermal apical membrane of the pulp to form the cavity of a pulp cap. During reconstitution the apex of the pulp and the apical membrane are restored. A single keratinized, thimble-shaped pulp cap is formed in each period of resorption from the apical membrane. During earlier stages of regeneration, although the pulp is steadily increasing in length, periodic resorption begins on the eighth day; but, as long as the apex of the sheath is intact (pinfeather stages), a series of delicate partitions only is formed across the apical cavity between the tip of the pulp and the closed apex of the sheath. These differ from the typical pulp caps and resemble in miniature the scala of the calamus.

The weight of pulp and of feather was determined at 2-day intervals from the thirteenth day of regeneration. From these figures it was calculated that the ratio of weight of pulp to that of feather produced is of the order of 3 to 1, and that the weight of pulp and feather required for a complete molt is of the order of 25 per cent of the total weight of the bird.

The circulation in the pulp is described for the first time, and remarkable adaptations for rapid growth of the feathers, periodic resorption of pulp, and maintenance of a constant pattern in the moving substratum of the pulp are discussed.

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ABBREVIATIONS USED IN ILLUSTRATION LEGENDS

a. arb. = apical arborization
a. c. = apical cavity
a. m. p. = apical membrane of the pulp
ax. art. 1 = axial artery in the papilla
ax. art. 2 = axial artery in the pulp
ax. pl. = axial plate
b. = barb; section of column of cells that will form the barb
b'bl. pl. = barbule plate
b. m. = barb membrane
cen. p. v. = central papillary veins
c. mu. = circular muscles of the wall of the follicle
c. p. c. = cavity of pulp cap
col. = collar
d. = follicular debris
ep. = epidermis
fol. = wall of follicle
fol. cav. = cavity of follicle
k. a. m. = keratinized layer of apical membrane
l. = basal reuniting loops of the pericentral sinuses from which central papillary veins arise
l. mu. = levator muscle of the feather
m. a. m. = middle layer of the apical membrane of the pulp
m. l. a. m. = Malpighian layer of the apical membrane of the pulp
pap. = papilla
p'c. sin. = pericentral sinuses
per. p. v. = peripheral papillary veins
p. m. = pulp membrane
pu. = pulp
pu. c. = pulp cap
r. = ridges
rh. = rhachis
r. z. = ramogenous zone
sh. = sheath
str. corn. = stratum corneum (forms sheath)
str. cyl. 1 = stratum cylindricum: the part in contact with the pulp destined to form the pulp membrane and the barb membrane
str. cyl. 2 = stratum cylindricum: the adjacent layers which form a barb septum
str. int. = stratum intermedium (forms feather)
v. = ventral

PLATE I

FIG. 4.—Transverse section, breast of White Leghorn cock; 19 days' regeneration. ×43. The level of the section is a short distance above the papilla (cf. Pl. II, Fig. 1; text Fig. 19; and Pl. IV, Fig. 20).

FIG. 5.—Part of Fig. 4. ×533. N.B.: The cells of the ridge bounded by the stratum cylindricum and the stratum corneum (sheath) constitute collectively the stratum intermedium.

PLATE II

FIG. 1.—Frontal longitudinal section through the base of a regenerating breast feather, White Leghorn cock; 19 days' regeneration. ×36.

FIG. 8.—Photograph of longitudinal section of stage I of pulp-cap formation, breast feather, Brown Leghorn capon; 25 days' regeneration. ×36. (Cf. text Fig. 7.)

FIG. 9.—Photograph of longitudinal section of stage II of pulp-cap formation, breast feather, Brown Leghorn capon; 25 days' regeneration. ×36. (Cf. text Fig. 7.)

FIG. 10.—Photograph of longitudinal section of stage IV of pulp-cap formation, breast feather, Brown Leghorn capon; 25 days' regeneration; keratinization, beginning in *m. a. m.*, does not correspond exactly to diagram, text Fig. 7, *IV*. ×36. (Cf. Fig. 7.)

PLATE III

FIG. 11.—Photograph of pulp caps showing incomplete resorption; from regenerating breast feathers, Brown Leghorn capon. × *ca.* 7.

FIG. 12.—Dried pulp caps showing barb septa in position. *A* = dorsal view showing rhachis depression in center; *B* = lateral view; *C* = another dorsal view. *A* and *B* = Brown Leghorn capon, breast, 27 days' regeneration; *C* = Brown Leghorn capon, saddle, 25 days' regeneration. ×6.

FIG. 13.—Dried pulp caps after loss of barb septa, breast feather, Brown Leghorn capon; 25 days' regeneration. *A* = three caps held together by barb membrane; *B* = two caps breaking apart. ×6.

FIG. 14.—Regenerating saddle feathers, Brown Leghorn capon; 33 days' regeneration, with persistent sheath and all pulp caps in place. × *ca.* 2.

PLATE IV

FIG. 20.—Photograph of basal portion of a bisected injected feather germ to show the pericentral sinuses and capillaries, breast feather, White Leghorn cock; 21 days' regeneration. ×44.

FIG. 21.—Photograph of basal portion of a bisected injected feather germ to show the pericentral sinuses and capillaries and one of the central papillary veins; breast feather, White Leghorn cock; 21 days' regeneration. ×44.

FIG. 22.—Apical arborization of regenerating breast feather of a juvenile White Leghorn, with complete pulp cap above it; stage II of resorption; dissection of injected specimen. ×44.

FIG. 23.—Apical arborization of breast feather, White Leghorn cock; 21 days' regeneration; stage IV of resorption as shown by structure of apical membrane; bisected injection. ×50.

PLATE I

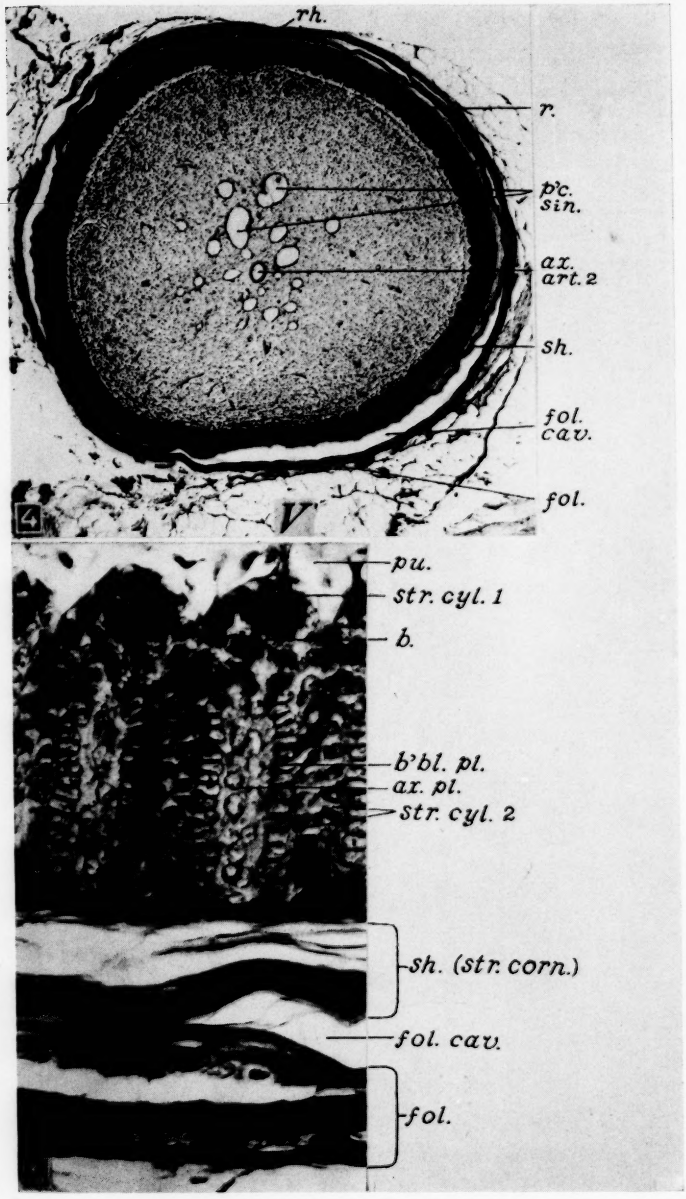

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PLATE II

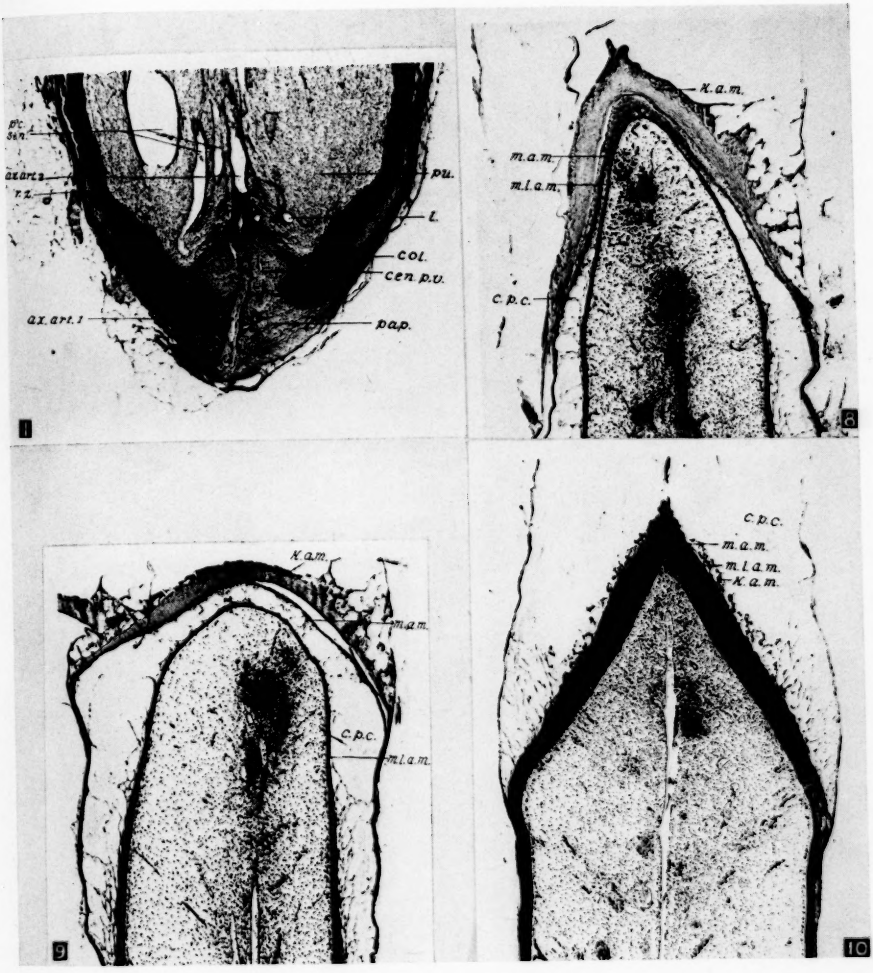

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PLATE III

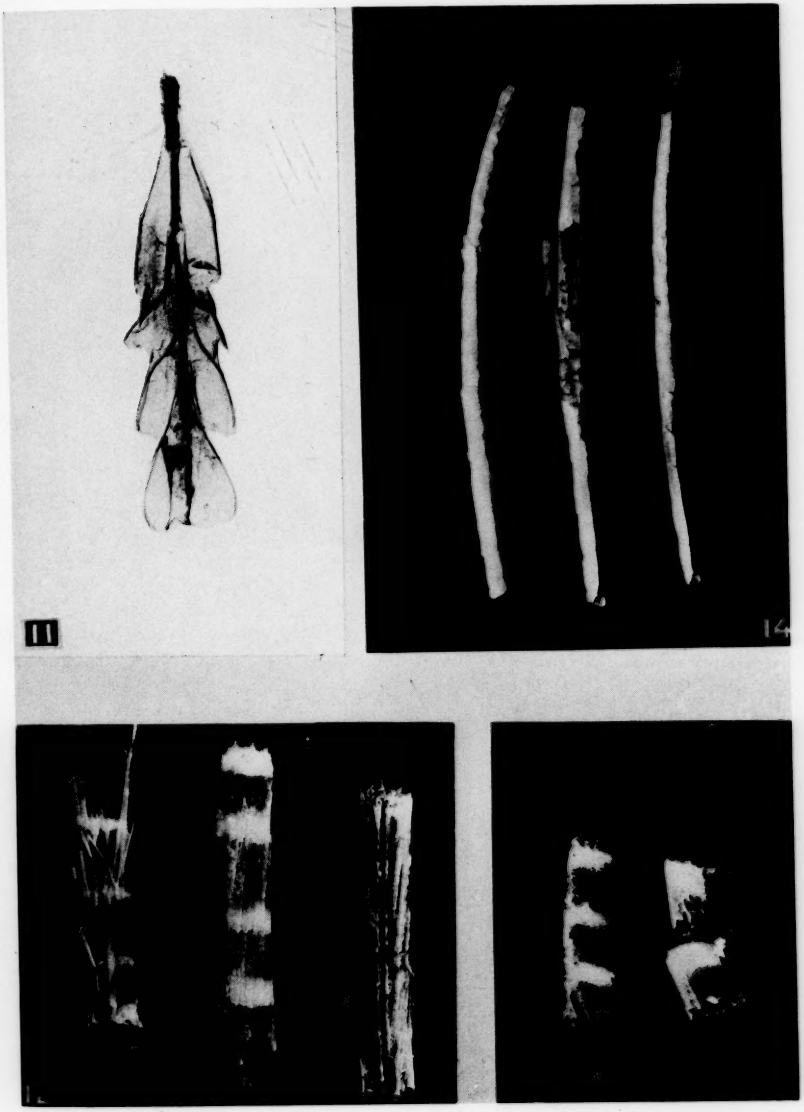

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PLATE IV

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THE CONTROL OF FEATHER COLOR PATTERN BY MELANOPHORES GRAFTED FROM ONE EMBRYO TO ANOTHER OF A DIFFERENT BREED OF FOWL

(One text figure; seven plates)

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THE present study had its origin in experiments designed to analyze the behavior of skin transplants made between chick embryos of genetically different breeds of fowl (Willier, Rawles, and Hadorn, 1937). By grafting a piece of skin ectoderm from the head to the wing—regions differing greatly in the arrangement, size, and shape of feathers—it was hoped that some light might be thrown upon the problem of the distribution of feather germs (i.e., their arrangement and spacing in tracts) and the localization and nature of the factors controlling their rate of growth, shape of feather produced, etc. The effects of the mesodermal substratum and humoral agents of the host on such developmental processes could thus be tested. In grafting the skin of one embryo to another of a different breed, a means is furnished for analyzing the expression of the different genetically determined potentialities of the combined tissues in the characterization of the feather.

When such host-graft combinations are made, an area of donor-colored down feathers is formed at and about the site of grafting in the great majority of cases. The down of these areas is replaced, after hatching, by juvenile contour feathers having the shape, rate of growth, and distribution in tracts characteristic of feathers occurring in corresponding regions of host-control chicks but invariably with the color or color pattern of the donor breed.

In seeking a solution to the problem of the manner of origin of the area of donor-colored feathers, it was found that this area did not arise solely by growth and spread of the implanted piece. It has been proved that the feathers of this area are derived structurally from host epidermis. The color, however, is produced under the control of melanophores derived from the donor tissue. Histological studies show that the implanted skin ectoderm, although incorporated at the implantation site, replaces little, if any, of the host epidermis of the affected area (Willier and Rawles, 1938*a*, 1938*b*, 1938*c*). Apparently at the time of implantation the skin ectoderm (from head region) was already "infected" with melanoblasts which had wandered in from the neural crest. From evidence now available, it is clear that these pigment cells migrate out from the implant into the host epidermis and the feather germs developing from it and produce the area of donor-colored feathers.

The purpose of the present paper is to present a general survey of the results obtained by grafting skin ectoderm and mesoderm from the embryo of one breed of fowl to another. In the analysis of the results, consideration will be given to several outstanding topics: (1) the respective roles that the donor and host play in feather characterization within the area of donor coloration; (2) the source and migration of the donor melanophores; (3) the breed-specific control of feather color or color pattern by the

donor melanophores, and the extent to which this control is independent of factors in the host; and (4) the peculiar constitution and behavior of the White Leghorn melanophores.[1]

BREEDS AND METHOD OF GRAFTING

For this investigation graft-host combinations between embryos of the following breeds were tested: Barred Plymouth Rock, Black Minorca, Buff Minorca, F_1 hybrid (from Barred Plymouth Rock ♀ × Rhode Island Red ♂), New Hampshire Red, White Silkie bantam, White Wyandotte, White Plymouth Rock, and Single Comb White Leghorn. For types of combinations tested see Table 1.

The method employed is that of grafting a piece of skin ectoderm or pure mesoderm from an embryo of one breed to another of the same age. The piece of skin ectoderm used is small, measuring 0.5–1.0 × 0.5 mm. The region from which the skin ectoderm is taken is first stained with Nile blue sulphate, so that the isolate can be easily seen during the subsequent handling and grafting. It is stripped from the body of the embryo by means of fine steel needles. During the isolation the embryo and pieces removed are kept until grafted in Locke's physiological salt solution, warmed to a temperature of 38° C. on a thermostatically controlled stage-warmer.

The source of skin ectoderm was usually the dorsolateral surface of the head anterior to the otocyst; but in a few cases it was taken from the wing bud, leg bud, or dorsal surface of a somite of the trunk region. To such pieces a small amount of mesenchyme always adheres. A histological study of pieces isolated from the head, however, shows that the number of mesenchyme cells is very small. When pure mesoderm was used for implantation, it was usually isolated from the limb buds.

The host embryo is prepared for receiving the implant by first sawing a window in the shell directly over the embryo, whose position is determined by candling. The shell over the window is removed, and enough of the shell membrane to expose the body of the embryo. The embryo is now viewed through a binocular dissecting microscope, and the site for transplantation located. This is usually the base of the wing bud; but in a few cases the graft was made to the dorsal surface of the head, to the leg bud, and to the tail bud. With very fine steel needles an opening is then made in the chorion and amnion over the selected site.[2] Through these openings an incision is now made with a glass needle in the skin ectoderm at the base of the wing bud, leg bud, or other locus selected for implantation. See Plate I, Figure 2. The isolated piece is carefully pushed into the incision—deep enough into the host mesoderm to anchor it in position. Often the free end is more or less in contact with the surrounding host ectoderm. The piece of shell is now returned to the window, sealed in position with melted paraffin, and the egg returned to the incubator.

The egg, during and subsequent to the operation, is kept on a nest of cotton on a Syracuse watch glass with the window surface uppermost. The majority of chicks hatch normally, but some have considerable difficulty in emerging from the shell and have to be helped out by carefully picking away the shell at the large end of the egg.

[1] We take this opportunity to express to Professor H. B. Hutt and Dr. J. H. Bruckner, of Cornell University, our appreciation of their friendly interest in this work and their co-operation in furnishing us with eggs from a cross between Rhode Island Red and Barred Plymouth Rock breeds.

[2] These membranes are usually stained with Nile blue sulphate to make them more easily seen. The staining is carried out very conveniently by means of an agar-coated glass rod dipped in a solution of the dye.

GENERAL STATEMENT OF RESULTS

The data obtained by grafting a piece of head skin ectoderm from embryos of one breed to the wing bud of another are summarized in Table 1. From an inspection of this table it will be seen, in the first place, that all possible types of combinations have been made between donor and host embryos of different breeds having either pigmented or white feathers. These are: (1) pigmented to white, (2) pigmented to pigmented, (3) white to pigmented, and (4) white to white-feathered breeds.

Secondly, skin ectoderm from embryos of breeds with pigmented feathers or with white feathers grafted to embryos of breeds having either white or pigmented plumage results in the formation of an area of donor-colored feathers on the wing and adjacent regions (see Figs. 4–10). Lastly, it will be noted that host-graft combinations of the various white-feathered breeds (Leghorn, Plymouth Rock, and Wyandotte) produce white, not pigmented, feathers. The donor-colored area, if produced, is indistinguishable from the host-colored feathers.

The table also shows whether or not an area of donor-colored feathers is produced in the host, and the frequency of occurrence. This is indicated for both the embryos which failed to hatch and the chicks which hatched.

In another, less extensive series of experiments with the same breed combinations, both the source and the site of transplantation were varied. Pieces of skin ectoderm and of pure mesoderm from the head, trunk (somite level), wing buds, and leg buds were grafted to the head, tail, and leg-bud regions of host embryos. At all ages tested (62–108 hours), donor skin ectoderm or mesoderm from the head or the trunk region produced an area of donor-colored feathers irrespective of where the transplant was placed. The results were briefly as follows: head skin ectoderm to tail bud, 14 positive, 6 negative; to leg bud, 2 positive, 1 negative. Skin ectoderm from the trunk region transplanted to the head, 2 positive, 1 negative; to the wing, 10 positive, 10 negative. When, however, the transplant was taken from the wing or leg buds, the age of the donor was of great importance in determining whether a positive or a negative result would be obtained. For example, wing skin ectoderm taken from donors 62–79 hours and transplanted to the head of a host embryo gave 17 negative and no positive results; but when the donors were 80–103 hours old, 9 negative to 13 positive cases were obtained. Similarly, wing skin ectoderm from donors 70–80 hours grafted to the wing region gave 11 negative and no positive cases, as compared with 3 negative and 7 positive cases obtained when the donors were 80–132 hours old. Also, leg-bud skin ectoderm from donors 94–103 hours gave 10 negative and no positive cases when transplanted to either the head or the wing-bud regions, while no negative and 2 positive cases resulted when the donor was 132 hours old.

Grafts of pure mesoderm gave results, in general, like those just described for skin ectoderm. Head mesoderm (72–100 hours) grafted to the wing gave 13 positive to 2 negative cases; mesoderm from the wing bud of donors 96–116 hours grafted to the host wing bud produced 93 positive and 95 negative cases (see Willier and Rawles, 1938*c*, Fig. 2).

Lastly, an examination of the results from all the various types of experiments in which the White Leghorn served as donor shows that it does not often produce an area of white feathers. So far, no positive results have been obtained with F_1 hybrid and New Hampshire Red host embryos, and very rarely with Barred Plymouth Rock hosts. Table 1 shows that, out of 18 cases in which White Leghorn head skin ectoderm was

TABLE 1

SUMMARY OF DATA ON GRAFTING HEAD SKIN ECTODERM TO WING BUD

Donor		Host						
					Area of Donor-colored Feathers			
					No. of Positive Cases		No. of Negative Cases	
Breed	Age (Hours)	Breed	Age (Hours)	Embryo Dead before Feathering	Embryo*	Hatched	Embryo*	Hatched
Black Minorca	68–76; 108	White Leghorn	68–76	7	8	13	3	5
Black Minorca	72, 114	White Wyandotte	72, 91	0	1	0	4	5
Buff Minorca	70–76	White Leghorn	68–75	3	13	6	5	2
Buff Minorca	73, 77	White Wyandotte	73, 77	2	1	2	0	1
Barred P. Rock	71–78; 82, 106	White Leghorn	71–84	17	26	15	7	9
Barred P. Rock	74, 77	White Silkie	74, 77	14	5	0	7	0
Barred P. Rock	76, 80, 98	White P. Rock	72–73	9	2	2	3	1
Barred P. Rock	73–76; 80	White Wyandotte	73–76	7	5	4	4	4
N.H. Red	75–76; 83	White Leghorn	72–76; 83	1	7	4	1	1
F_1 hybrid	63–101	White Leghorn	65–100	36 (9)†	69 (9)†	45	16 (3)†	16
Black Minorca	72–76	Barred P. Rock	70–76	1	0	4	11‡	10
Barred P. Rock	77	Black Minorca	99	1	0	0	1‡	1§
Barred P. Rock	73–74	Buff Minorca	76–77	1	3	0	1	1
N.H. Red	71–79	Barred P. Rock	71–79	15	4	7	9	2
Barred P. Rock	71–82	N.H. Red	71–82	15	1	4	4	4
White Leghorn	71–75	Black Minorca	71–76	10	9	1	10	5
White Leghorn	71–76	Buff Minorca	72–76	2	1	1	6	4
White Leghorn	70–80; 91	Barred P. Rock	60; 70–84	9	2 (trace)	0	11	5
White Leghorn	72–76; 88–92	F_1 hybrid	72–76; 88–92	(14)†	0	0	(11)†	0
White Leghorn	72–78; 83	N.H. Red	72–78; 83	12	0	0	14	13
White Wyandotte	74–76	Black Minorca	74–76	0	2	4	0	0
White Wyandotte	75	Buff Minorca	74	1	1	3	0	0
White Wyandotte	73–83	Barred P. Rock	73–80	12	8	8	4	2
White Wyandotte	72–73	F_1 hybrid	72–78	2	0	3	2	2
White Silkie	70, 80–84	Black Minorca	70–75	10	11	3	1	0
White Silkie	70–80	Barred P. Rock	70–80	8	2	9	4	4
White Silkie	70–80	Barred P. Rock	70–80; 102–107	8	2	9	4	4
White Leghorn	82	White Wyandotte	73	0	0	0	1	1
White Leghorn	71–76; 86, 92	White P. Rock	71–76	2	0	0	8‖	12
White P. Rock	62, 63	White Leghorn	62, 63	3	0	0	6	3
White Wyandotte	70–79	White Leghorn	70–78	5	0	0	11	16

* Fully feathered; died before or at time of hatching.

† Numbers in parentheses are those killed and examined before hatching.

‡ Down feathers of host and donor indistinguishable.

§ Died before juvenile plumage emerged.

‖ Down feathers often bluish gray, a characteristic also found in control White Plymouth Rock embryos.

transplanted to Barred Plymouth Rock, only 2 showed traces of white down on the right wing. Two other positive cases from many trials have been obtained with Barred Rocks—one from a transplant of skin ectoderm from the wing base of a $7\frac{1}{4}$-day-old embryo and another from a transplant including the dorsal part of a somite and neural tube from the posterior region of a 78-hour embryo (see Fig. 11). With Black and Buff Minorca hosts the White Leghorn implant produced an area of white feathers on the wing with higher frequency, although never very extensive (head skin ectoderm to wing bud gave 12 positive to 25 negative; skin ectoderm overlying the somites of a 72-hour donor gave traces of white feathers in 3 out of 3 trials).

THE AREA OF DONOR-COLORED DOWN FEATHERS

The area of donor-colored down feathers has been produced in various regions of the body, depending upon the site of grafting. When grafted to the base of the right wing bud, donor-colored plumage develops usually on the entire surface of the wing (see Figs. 4–10). In some cases it covers either the humeral or the ulna-radial portions only. In still others the area is more extensive, covering not only the entire wing but adjacent parts of the breast, neck, rump, and thigh of the operated side. In these cases the donor-colored plumage does not often extend across the mid-ventral or mid-dorsal line (figs. 5 and 6). When the spread is greatest, a single area may include the humeral and alar tracts on the wing, the breast tract, a portion of the spinal tract, and the femoral and crural tracts of the leg. If the implant is pushed deeply into the body wall at the base of the wing bud, the donor-colored plumage may be confined to the breast. In general, the path of extension of the effect is toward the tip of the wing and ventrally to the mid-line of the breast.

The same donor effect may be produced on any region of the body irrespective of the source of the implant. If head skin ectoderm of a Barred Plymouth Rock or Buff Minorca embryo is placed into the tail bud of a White Leghorn embryo, the entire uropygium and adjacent regions of the back develop donor-colored feathers. Leg bud, wing bud, or back skin ectoderm (Barred Plymouth Rock, F_1 hybrid) grafted on the head of a White Leghorn produces a relatively small localized area of black down (Fig. 3). When head skin ectoderm is implanted to the base of the leg bud (F_1 hybrid to White Leghorn), an area of black down covers the thigh and shank of the leg and adjacent parts of the body.

In size and distribution the down feathers of the donor-colored area are identical with those occurring in corresponding regions of either the control embryos or the unoperated side (left) of the same embryo. Feathers on the wing have the form and arrangement characteristic of primaries, secondaries, and coverts.

A microscopical examination of the down feathers of the "graft" area shows that the barbs and barbules are entirely donor colored except those occurring at the margin. Here they are mosaics of donor- and host-colored barbs and barbules of variable proportions. The barbs and barbules are found to contain pigment granules of the donor variety when a pigmented breed is used, and no pigment granules when the donor is a white breed. The same observations have been made on the downy barbules of donor-colored feathers of the juvenile plumage and will be described more completely below.

REPLACEMENT OF THE DOWN WITH JUVENILE PLUMAGE IN THE AREA OF DONOR COLORATION

After hatching, the donor-colored down feathers of the "graft" area are gradually replaced with contour feathers of the juvenile plumage. These have the color or color pattern of the donor breed (see Figs. 11–20). Skin ectoderm or limb-bud mesoderm of a Barred Plymouth Rock donor will bring about the formation of black and white barring pattern in the contour feathers of the wing, breast, etc., of a White Leghorn host. New Hampshire Red grafted to White Leghorn or Barred Plymouth Rock produces red feathers with black markings, as in the control. Feathers produced when F_1-hybrid embryo is donor to White Leghorn host show sex-linked differences in color pattern. When the donor implants come from a male embryo, a barring pattern results; when from a female nonbarred feathers either solid black or with varying amounts of red pigment are formed (Fig. 20). These patterns developed on hosts of either sex and resemble very closely the male and female patterns found in donor control chicks of the same age.

The contour feather may be entirely donor colored or partly donor and partly host colored—i.e., mosaics of donor and host colors. Both types may appear on the same chick; in some, the mosaic type only occurs. There are several types of mosaic feathers. In the commonest of these the distal portion of the vane and shaft is the color of the donor, and the proximal portion is host colored (Figs. 21–23). The transition between these portions of the feather is quite sharp, forming a line roughly transverse to the shaft. The proportion of donor- and host-colored regions varies with the sequence in origin of the contour feathers. The amount of the donor-colored portion is greatest in the primary and secondary flight feathers which arise first and least in those arising later (Fig. 20). The amount may be still less in the coverts and breast feathers, which arise still later than the primaries and secondaries. The donor influence on color production which thus ceases before the emergence of the juvenile plumage is completed never reappears.

A second type of mosaic feather is found which differs from the one just described in that the transition between donor and host coloration is more gradual and somewhat irregular. A variable number of patterns is exhibited among the wing and breast feathers: (1) those with the shaft donor colored either throughout its length or distal portions of varying lengths; (2) feathers like the foregoing except for the bases of the barbs, which are donor colored also; (3) feathers with donor coloration extending in a proximal direction farther on one side of the vane than on the other (in these the transition zone is somewhat broad, donor and host coloration intermingling).

A third type of mosaic feather may be designated "alternating," since the donor- and host-colored areas in the vane tend to alternate. The donor color merely temporarily disappears and then reappears. The cessation in the formation of the donor color may take place once or several times during the outgrowth of the feather. A variety of patterns has formed among the flight feathers, wing coverts, and breast feathers of the juvenile plumage. The following few cases will illustrate the phenomenon. The first, a secondary flight feather (No. 1) has its distal one-fifth barred like the host (Plymouth Rock), while the remainder is white like the donor (Silkie Bantam); upon plucking, this feather was replaced by one entirely barred, like the host. A second example is that of an under wing covert. The distal one-fifth of this feather is donor (F_1 hybrid ♀); the

second fifth is host (White Leghorn); the third fifth, donor; the fourth fifth, host; and the proximal fifth, donor. A third case is that of a secondary flight (Nos. 1 or 10). In this the distal portion of the vane (half or fourth) is entirely donor (F_1 hybrid ♀); the middle portion, host colored (White Leghorn) except for the shaft and bases of the barbs, which are donor colored; the proximal portion, entirely donor-like.

A fourth type of mosaic feather found is unilateral with respect to color. The donor color is confined to one side, either on the inner or on the outer vane. In such a feather, shown in Figure 24, the entire inner vane and shaft may be mostly black, like the host (Black Minorca), and the outer vane entirely white, like the donor (Silkie Bantam); or the distal half of the inner vane (i.e., sixth primary) may be barred like the donor (Plymouth Rock) and the proximal half red like the host (New Hampshire Red), while the outer vane is entirely host colored. In some feathers (i.e., ninth secondary), the vane is entirely donor colored (White Silkie) except for the bases of the barbs of the inner vane, which are host colored (Black Minorca). In addition to these, other feathers are found in which the regions of donor color, although confined to one side, are smaller and of variable shape.

A microscopic examination of barbule cells from the downy region of the contour feathers shows, as did the down feathers, the presence or absence of pigment granules, depending upon whether the donor was from a pigmented or nonpigmented breed (see Figs. 26–37). When pigmented breeds, such as the Barred Plymouth Rock and the Black Minorca, are donors to White Leghorn hosts, the barbule cells contain black granules characteristic of these breeds. In Figure 33 White Leghorn host barbule cells containing pigment granules of the Black Minorca are seen. These granules are of the same form as those seen in the control Minorca cells. Similarly, when Barred Plymouth Rock or New Hampshire Reds are used, the granules occurring in the White Leghorn host are, respectively, of Barred Plymouth Rock and New Hampshire Red types (Figs. 27 and 30). Their distribution is the same in the host feathers as in the donor control feathers. When white breeds, such as Wyandotte, Silkies, and Plymouth Rocks, are donors, the barbs and barbules of the white host feathers, as in the donors, contain few or no pigment granules (Fig. 37). Likewise, White Leghorn implants in Black and Buff Minorca hosts may bring about the formation of a few white down feathers containing some pigment granules, more or less localized at their bases, as in control donors.

The various contour feathers developed in the "graft" area have the same type and arrangement as feathers in corresponding positions of host controls of the same age or on the unoperated wing (left) of the host (Fig. 4). Those on the wing are arranged in definite tracts containing the primaries on the hand; secondaries and coverts, etc., on the forearm. On the breast they have the distribution characteristic of the ventral tract. Their shape and size are always peculiar to the tract or a particular region of the tract in which they occur. In no case do the feathers of the donor-colored area resemble in shape and distribution the feathers of the head, limb, or trunk regions of the embryo from which the skin implant was taken. For example, head skin ectoderm, when implanted to the wing, does not replace host skin and produce feathers like those occurring on the head of control donor chicks—i.e., small feathers arranged in a number of small tracts. The feathers are entirely of the host wing type.

Likewise, the rate of growth of the donor-colored feather is that of the host. The daily rate of growth of certain primaries and secondary flight feathers has been measured and found to follow exactly that found for corresponding feathers on the unoperated left wing

(see Fig. 4) or on host controls of the same age. Figure 1 shows a graph in which the rate of growth in length of the fourth and fifth primaries, having the color pattern of the Barred Plymouth Rock donor, a slow-feathering breed, is found to be practically identical with that of the fourth and fifth primaries of the left wing of the White Leghorn host, a fast-feathering breed. The rate of growth in length of these feathers in a Barred Plymouth Rock control chick is distinctly slower than in the White Leghorn. Also, the third and fourth red primary feathers colored by a New Hampshire Red im-

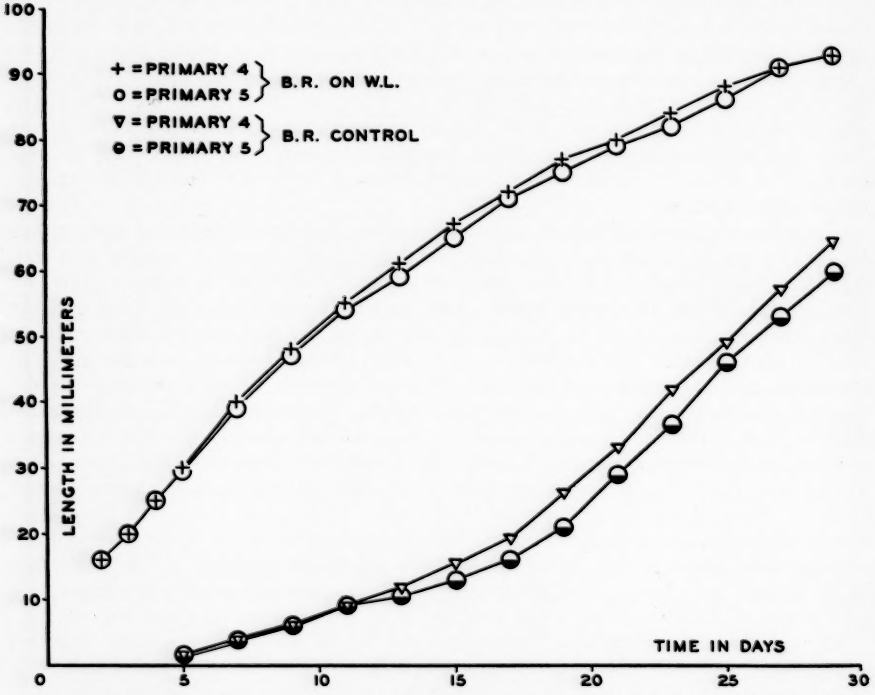

FIG. 1.—Comparison of the rate of growth in length of barred (donor pattern) primary flight feathers Nos. 4 and 5 of a White Leghorn host with that of the same feathers of a Barred Plymouth Rock control The donor colored feather has the White Leghorn and not the Barred Plymouth Rock rate of growth.

plant on a Barred Plymouth Rock host develop at the Barred Plymouth Rock rate of feathering. In the control New Hampshires these feathers grow in length somewhat more rapidly than in the control Barred Plymouth Rock.

REPLACEMENT OF JUVENILE BY ADULT PLUMAGE

The donor-colored juvenile plumage of the "graft" area is gradually replaced with adult plumage as molting takes place. Usually with this molt the new feather which emerges is entirely host colored, the donor color having completely disappeared. The order in which this takes place in the flight feathers of donor color on White Leghorn

hosts is a very regular one, agreeing quite precisely with the order described by Warren and Gordon (1935) for the normal White Leghorn. The primaries are molted in regular sequence, beginning with No. 1, adjoining the axial feather, and progressing outward to No. 10. The secondaries are dropped in the reverse order, beginning with No. 2 and progressing inward. Secondary No. 1 and the axial are molted considerably later. The new adult flight feathers emerge in the same order in which the juvenile ones are dropped, but usually with the color of the host. The sequence in the shedding of the coverts and breast feathers was not followed.

In certain exceptional cases some of the primaries and secondaries are replaced by adult feathers which are mosaics of donor- and host-colored parts. In one feather (first primary) of the adult plumage of a White Leghorn chicken which received an implant of Barred Rock tissues, the outer vane has the Barred Rock pattern, whereas the distal half of the inner vane is entirely white (hostlike), and its proximal half is barred. The shaft of the feather is barred except the white half next to the white vane. Another feather (fifth secondary) is nearly identical with the one just described. Still another type of mosaic adult feather is shown in Figure 25, where the host is White Leghorn and the donor Black Minorca. Here the feather is entirely black, like the donor, except for two symmetrically arranged host-colored (white) areas in the inner and outer vanes at the tip (this is possibly an injury effect brought about by plucking; see Kuhn, 1932). A third type of mosaic adult feather has the donor color (black) largely confined to the shaft and the bases of the barbs of both outer and inner vanes. In some feathers the black pigment may extend the entire length of, or only part way down, the shaft. Toward the base of the feather the black pigment not only may be confined to the shaft but may occur on one side of it, the other side being white or host colored. Finally, it may be noted that, as far as our studies go, when such mosaic feathers are replaced the new (second adult) feathers are entirely host colored.

FATE OF THE IMPLANTED SKIN ECTODERM

Attempts have been made to follow the fate of the implanted head skin ectoderm and its adhering mesenchyme in the host embryo. At successive intervals after implantation, ranging from $5\frac{1}{2}$ to 72 hours, the host embryo was prepared for histological study in the usual manner, and the right wing bud was carefully examined for the implanted tissue. Twenty-seven cases have been examined. In the majority of these, 21 in number, the host was White Leghorn and the implant Barred Plymouth Rock; in 4 cases the host was Barred Plymouth Rock and the donor White Leghorn; and in 2 instances Barred Plymouth Rock was host to New Hampshire Red tissue.

The results of this study show almost invariably at the implantation site the presence of a piece of the skin ectoderm of the implant adjacent to which is a mass of loose, light-staining mesenchyme. The piece lies deep in the limb mesoderm, especially during the early hours after implantation. It is usually continuous at the surface, with epidermis covering the original incision. How much of the surface epidermis is of host and how much of donor origin cannot be ascertained, owing to the absence of distinguishing features.[3] The epidermis beneath the surface may be folded upon itself in the form of a

[3] On the basis of the interpretation reached in this paper, a feather developing from any persisting epidermis would be structurally like the donor breed but colored according to whether the feather germ was invaded by donor or host melanophores. Of all the donor breeds tested, only the White Silkie has feathers distinguishable by structural features from the others. The Silkie type of feather has not been found in the "graft" area, indicating the absence of persisting donor epidermis.

vesicle, or its free end may terminate blindly in the wing mesoderm. The deeper portions of the skin ectoderm, particularly when entirely surrounded with mesoderm, become disorganized; its epithelial character is lost, and the cells intermingle with, and are indistinguishable from, the mesodermal cells of the wing bud. In a few instances the implant is situated near the surface of the wing and may protrude through the original incision and fuse with the amnion. In such cases it is inferred that the implant is in the process of being lifted out.

The mass of loose, light-staining mesenchyme of the "graft" region contrasts sharply with the dense, dark-staining limb mesoderm. The source of this mesenchyme is uncertain. It may arise from the mesenchyme introduced with the head skin or from both donor and host cells resembling a tissue akin to scar tissue in the adult. In no case can donor mesenchyme or other types of cells be identified.

It is apparent from this study that the implant remains localized at the base of the wing bud, although the limb has increased considerably in length. The growth of the wing bud is apparently entirely distal to the site of implantation. There is no evidence that the skin ectoderm of the implant spreads over the wing, replacing the host epidermis of the donor-colored feather.

Finally, it should be noted that there is no difference, so far as these studies go, in the behavior of White Leghorn implants on Barred Plymouth Rock from that found in reciprocal combinations of these breeds. The failure of the White Leghorn grafts to produce donor-colored feathers in F_1-hybrid and New Hampshire Red hosts must therefore be explained on other grounds.

DISCUSSION

Manner of origin of the area of donor-colored feathers.—Both donor and host appear to play a role in feather characterization within the graft area. Structurally, the feathers are of host epidermal and not of graft origin. This is shown by several lines of evidence. First, the spread of the donor-colored effect over the entire wing and to adjacent feather tracts on the breast, back, and even to the thigh in some cases, is too extensive to regard the implant of skin ectoderm as the source of the epidermal cells of the feather germs. Second, a histological study of the fate of the skin ectoderm made at successive intervals after implantation reveals that it does not spread over the wing, replacing the host epidermis of the graft area, but remains localized at the base of the limb bud, the site of grafting. Apparently only a small surface portion heals in, becoming continuous with the surrounding host epidermis; the deeper portions of the implant become disorganized, the epithelial character of the ectoderm is lost, and the cells intermingle with, and become indistinguishable from, the mesodermal cells of the wing bud. The growth of the wing bud is apparently largely distal to the site of implantation.

In the third place, the donor-colored contour feathers of the juvenile plumage have the same shape, rate of growth, and distribution in tracts as those found in corresponding regions of host control chicks (see figs. 1 and 4). In these particulars they never resemble feathers of the head region, which might be expected, assuming that the donor epidermis retained its head qualities and produced them. Of particular importance in this connection are the results of implanting Silkie Bantam skin ectoderm into Black Minorca or into Barred Plymouth Rock hosts. In these cases, had the Silkie ectoderm formed the structural elements of the donor-colored feathers, such feathers would, barring the possibility of host effects, have possessed the defects characteristic of the donor breed, such as

missing barbicels in most feathers, frayed margins of the vane in the primary and secondary flight feathers, etc. Such was not the case. In every respect except color, the feathers which formed in the graft area were identical with host control feathers.

A fourth consideration is the fact that a donor-colored area of feathers produced by an implant of limb-bud mesoderm or somite mesoderm (including neural crest cells) (Watterson, 1938) has characteristics identical with one produced by an implant of skin ectoderm. In the former case the host epidermis undoubtedly forms the feather structure, since no donor ectoderm was transplanted.

Finally, conclusive evidence that the donor-colored feather arises structurally from host epidermis following skin-ectoderm implantation has been furnished by grafting Robin into White Leghorn, birds belonging to two widely separated orders (Rawles, 1938 and 1939). A microscopical examination of the proximal and distal barbules of the inner and outer vanes and of the downy barbules of Robin-colored contour feathers of the host shows that the feathers are definitely White Leghorn in structure. Only the color of the feather is Robin, owing to the presence of pigment granules of a specific Robin variety.

The color or color pattern of the feathers in the graft area, on the other hand, is produced under the control of the donor breed. In all the various combinations of breeds tested, the color or color pattern of the feather has been that of the donor breed. This is true irrespective of whether the donor belongs to a pigmented or unpigmented breed. The only exception to this rule occurs when White Leghorn is donor to F_1-hybrid and New Hampshire Red hosts, in which cases white feathers fail to develop in the "graft area." For other peculiarities in the behavior of White Leghorn implants see page 181.

From these findings on the contribution made by donor and host, the interpretation is reached that the feather of the graft area is the product of the joint action of host feather germs and some influence arising from the implant of donor tissue. In other words, the structure of the feather is the product of the epidermal collar of a host feather germ, but its color or pattern is under the control of the implanted cells.

That the feather coloration is controlled in some way through the agency of melanophores derived from the donor was first suspected from the well-known fact that feather pigmentation in the fowl is associated commonly with the activity of melanophores in the epidermis of the feather germ. These, according to Strong (1902 and 1917), Greite (1934), and others, are branched cells which deposit or "feed" melanin pigment granules directly onto the surface (Greite) or into the interior (Strong) of the epidermal cells of the developing shaft, barbs, and barbules.

Several lines of evidence support the theory that the donor melanophores have actually migrated into the epidermis of the host feather germ, where they deposit pigment granules onto the barbs and barbules. In the first place, Watterson, working in this laboratory, has observed dermal melanophores entering the epidermis prior to the formation of feather germs in normal chick embryos of the Barred Plymouth Rock breed. Secondly, that melanophores do normally arise outside the epidermis and migrate into it is clearly shown by transplantation experiments. Our first clue as to their extrinsic origin came from experiments designed to test the capacity of skin ectoderm from various regions of the embryo to produce a donor-colored area in host embryos. It was noted for donors incubated less than 79 hours that skin ectoderm from the wing bud and from over the somites gave, respectively, negative and positive results. Later, after 79 hours, ectoderm from the wing bud, as well as from the somite region, gave positive

effects. Ectoderm from the leg bud did not give positive results until still later, i.e., after 100 hours. In a similar manner Watterson (1938) tested the pigment-producing potency of mesoderm cells from the limb bud and from somites. He found that the pigment-producing cells are present in the segmental plate and somites of 33–72-hour donors and appear in the wing bud sometime between 72 and 92 hours. He further showed that these pigment-producers appear still later in the leg bud—viz., in a donor of about 99 hours. From these two lines of results the conclusion is reached that (*a*) the source of the pigment cells is in the region adjacent to the neural tube, whence they migrate laterally into the limb buds and into overlying ectoderm, and (*b*) their outward spread is later in posterior than in anterior levels.

Eastlick (1939*a* and 1939*b*) reached a similar conclusion from a different type of analysis. He grafted a limb bud from 24–30 somite embryos of pigmented breeds (Brown Leghorn or Barred Plymouth Rock) to the coelom of White Leghorn or of pigmented host embryos of the same somite number and found that, if the bud was isolated so as to include all material (except entoderm) up to the neural tube, it developed pigmented feathers in the graft. If, however, the limb bud was isolated from the body at the level of the nephrotome, it produced down feathers without pigment. Thus, in the absence of pigment cells located in the region of the neural tube a feather germ of a pigmented breed can produce only white feathers.

Evidence of a similar nature is furnished by grafting wing buds of different ages to the coelom or to the chorio-allantois (Ris, 1940). A wing bud isolated from Barred Plymouth Rock embryos of 72-hours or earlier and grafted to these sites in either Barred Plymouth Rock (in case of coelomic grafts only if attached to mesentery) or White Leghorn hosts produces white down feathers only, no melanin pigment being formed. Similar transplantations of wing buds of older embryos (90 hours and over) give black down feathers in the graft. By means of a histological examination Ris showed further that melanophores are present in the epidermis of the feather germs of the latter but not in the former.

Lastly, crucial evidence that the donor melanophores have entered the epidermis of the host feather germ is furnished by the discovery of melanin granules of a shape, size, and color peculiar to the donor breed in the barb and barbule cells of the host feather. Thus, the conclusion is reached that the breed-specific melanophores have migrated away from the donor implant and have entered the feather germs of the host, where they deposit breed-specific granules.

The question next arises as to the source in the normal embryo of the melanophores which appear to migrate into the epidermis of the feather germs of limb buds and other body parts. The evidence discussed above on their extra-epidermal origin and lateral migration from the neural tube region, together with the work of Du Shane (1935, 1938) and others on the source of chromatophores in amphibians, leads to the hypothesis that the neural crest furnishes the melanophores in the chick. Reasoning from analogy, Dorris (1936, 1938, and 1939) was the first to suspect that the neural crest in the chick, as in the amphibian, embryo is the source of the melanophores. She consequently set about testing the capacity of the neural crest region in the chick to produce pigment cells. She first found that melanophores differentiate in explants of the neural crest region taken from embryos of several breeds of fowl. Later she showed that, when the neural crest is grafted to a host embryo, as in our experiments, it has the capacity to produce an area of donor-colored down feathers.

That the neural crest is the exclusive source of the epidermal melanophores has not been shown. The controls used by Dorris are inadequate for showing this, since little or no attempt was made to correlate pigment production in explants or in grafts with the order of appearance and development of the neural crest in the donor embryo. For example, ectoderm lateral to the somite or to the neural crest, if taken from one level in the anteroposterior axis, may give positive results, while from another level only negative results. Furthermore, the removal of the entire neural crest of an embryo is no assurance that pigment cannot form in isolates taken from levels of the axis where this structure has not yet formed. By transplanting parts from various levels of a Barred Plymouth Rock embryo into the coelom of a White Leghorn host embryo, Ris (1940) has shown that a definite correlation exists between the capacity of an isolate to produce pigment and the morphological development of the neural crest. Only those isolates known to contain a neural crest, the region from which it arises, or its migrating cells are able to produce melanophores.

Recently the pigment-producing capacity has been traced to presomite blastoderms, stages prior to the formation of the neural crest. Quite independently Eastlick (1939*a*) and Rawles and Willier (1939) tested pieces from various regions of blastoderms of the Barred Plymouth Rock breed for their capacity to produce donor-pigmented feathers in White Leghorn hosts when placed in the wing or into the coelom. It was found that the blastodermic area capable of producing donor-colored plumage in the host chick coincides with the area known to form nerve tissue (both central nerve tissue and ganglia) in chorio-allantoic grafts (see Rawles, 1936), being much more extensive than the prospective neural-crest region, as indicated by vital staining. For a more complete account see Rawles (1940). The wide distribution of the pigment-producing capacity characteristic of the presomite blastoderms becomes restricted to the neural-crest region in the somite stages. The time when this change takes place, as well as its manner, are problems of considerable significance.

Having shown that the implant is the source of the melanophores of the donor-colored area, the question now arises as to what factors control the degree, paths, and direction of their spread in the body of the host. In general, when placed at the base of the wing bud of a 70–75-hour host embryo, the path of their extension is toward the tip of the wing and ventral to, but usually not across, the mid-line of the breast; also, posteriorly onto the back and thigh and anteriorly to a much lesser extent. The spread, as a rule, does not extend dorsally across the mid-dorsal line. When placed into the tail bud, the melanophores become distributed in the tail and adjacent regions of the chick. It is evident, particularly for the wing level, that more or less definite paths are followed. That these are the same paths followed by melanophores in their migration outward from the neural crest in normal development seems probable. Although melanophores are migratory cells, some influence of an unknown nature appears to direct their movements along certain lines. Such a directive influence may be effective in causing them to leave the skin-ectoderm implant and enter the tissues of the host.

It is clear, from grafting experiments cited above, that the host melanophores do not enter the wing bud before approximately 79 hours. The donor melanophores, which are grafted prior to this time, consequently have an opportunity to migrate into the wing bud before those of the host come in. That they do migrate in and invade the developing skin and feather germs before the host melanophores do is shown by the result that feathers with the coloration of the donor are formed first. Apparently, at

first the donor melanophores occupy all the available places in the ectoderm of the skin and feather germs, thus excluding those of the host, which come in later. As the donor melanophores are used up during the process of pigmentation of the feather, they are replaced by those of the host.

The extent of the spread of these cells apparently depends roughly upon the quantity of melanophores introduced in the implant. In general, small implants, either of skin ectoderm or mesoderm, have a tendency to produce less spread than large ones, i.e., the spread produced by a small implant may extend onto the forearm or onto the breast tract, whereas that produced by a large implant may extend over the entire wing and to the mid-ventral line of the breast (see Figs. 5 and 6). Also, skin ectoderm from the region over a somite produces a more extensive spread than skin ectoderm from the head. Such a difference is expected on the basis of a time difference in the development of these two regions of the embryo. In the head, the older region, the neural crest, as such, has already disappeared; and the melanophores are somewhat widely distributed, being within most of the skin ectoderm and subjacent mesenchyme of the head (less mesenchyme adheres to the isolated skin ectoderm from the head; hence fewer melanophores may be carried over in the implant). In the somite region at the stages used, the neural crest cells lie immediately beneath the skin ectoderm. Here the potential melanophores are still concentrated.

The age of the host seems also to be a factor in controlling the degree of spreading. This is indicated by the results of a few preliminary experiments in which the host was from 1 to 2 days older than customarily used. Although implants of the usual size were used, the donor-colored area formed on the wing was generally smaller than in younger hosts. In some cases the donor pigment was in the skin but not in the feathers. From this result it is inferred that the conditions in the wing region of older hosts are less favorable for extensive migration and for migration along certain paths. At the time of implantation the melanophores of such hosts have, in all probability, migrated to some extent in the direction of their definitive positions in the wing bud. Also, it has been found that melanophores spread much less when implanted on the head than on the wing and leg buds of 72-hour host embryos (cf. Figs. 3 and 5). This is attributed to differences in developmental age, the head region having passed through the stage when conditions are most favorable for spreading.

Other conditions within the host, apart from age, affect the degree of migration of melanophores. The same kind of melanophore, implanted in nearly equal quantities to hosts of different breeds, generally shows less spread in Barred Plymouth Rock, White Wyandotte, and New Hampshire Red than in the White Leghorn or the Black Minorca hosts. In the latter two hosts the spread generally may be described as being very extensive. Furthermore, Barred Plymouth Rock melanophores in a White Leghorn host spread to the tip of the wing or onto the breast, whereas in normal control Barred Plymouth Rock embryos or chicks, these regions are covered with white down feathers, which later become pigmented and ultimately barred. The nature of these host differences which favor or restrict the extent of migration of melanophores is a problem for further investigation.

It is also possible that the different kinds of melanophores, owing to some peculiarity in their constitution, vary in their ability to migrate. This is indicated for the White Leghorn melanophore since, even though large numbers are implanted, the spread is

much less extensive, irrespective of the host used, than when other melanophores are introduced. For a further discussion of this matter see pages 193–97 ff.

Breed-specific control of color pattern in host feathers.—The color or color pattern of the feathers in the invaded area is specifically in accord with the genotypic composition of the donor breed. This is shown in various ways. (1) A Barred Plymouth Rock implant produces a barring pattern in host contour feathers of nonbarred breeds such as the New Hampshire Red, White Leghorn, and Black Minorca (see Figs. 12 and 13). Furthermore, two types of barring pattern occur, one being generally somewhat darker than the other. In the darker pattern the black bars are wider and darker than in the lighter one. These differences parallel closely sex-linked differences in plumage found in donor control chicks of the same age, where the feathers of the female are somewhat darker than those of the male. It becomes apparent, therefore, that a melanophore from a female donor which has only one gene for barring produces a darker-colored host feather than one from a male which has two genes. The sex of the host apparently has no effect on the result. A more complete analysis of this matter is proposed for a separate paper. (2) Implants from male and female F_1-hybrid embryos produce, respectively, barred and nonbarred (Fig. 20) contour feathers in a White Leghorn host, irrespective of its sex. (3) An implant of a New Hampshire Red brings about in White Leghorn or Barred Plymouth Rock hosts an irregular black-and-red pattern in the contour feathers such as occurs in donor control feathers (Fig. 14). (4) A Black or Buff Minorca implant produces in the contour feathers of White Leghorn or of Barred Plymouth Rock hosts a solid black or buff coloration (Figs. 15 and 16). (5) The white donor breeds, including the White Leghorn produce in certain cases white feathers in pigmented hosts (Figs. 11, 17, 18, and 19).

The color and color pattern appear to be directly controlled through the agency of melanophores. The melanophores from pigmented breeds deposit melanin granules peculiar to the donor into the epidermal cells of the shaft, barbs, and barbules of the host feather. These granules are of a specific shape, size, and color, being characteristic of those found in the donor control breeds (Pl. VII).

When Black Minorca and Barred Plymouth Rock furnish the melanophores, black rodlike granules, characteristic of these breeds, occur in the feather irrespective of the breed of host (Figs. 27, 28, 33, and 34). Buff Minorca melanophores deposit yellow-colored granules which are smaller and rounded in form. Grafts of New Hampshire Red give a result of special interest. The host feather has both red- and black-colored areas, as in the donor control chicks. In these areas are found, respectively, red (phaeomelanin) and black (melanin) granules, which differ strikingly in size and shape. The black ones are rodlike, resembling Barred Plymouth Rock granules, except for a slightly greater length. The red ones are small globular granules (Figs. 29–31). According to Ladebeck (1923), pigment granules identical with these are found in the red and black regions of feathers of the Rhode Island Red, a strain from which the New Hampshire Red was apparently derived. This observation suggests the presence of two specific types of melanophores in the New Hampshire Red.

If two kinds of melanophores have already differentiated prior to the formation of the feather germ, it will be necessary to assume the presence of some differential factors controlling their distribution to the red and black areas in the feather vane. It seems more probable that the melanoblasts, at the time of their invasion, are relatively un-

differentiated cells. They may have the dual capacity for differentiating into either red or black melanophores, the direction being determined by regional differences in physiological activity within the the epidermal substratum of the feather germ. Whether such melanophores are stable and incapable of changing one into the other is an interesting question. Raper (1932) has shown that various intermediate substances are formed in the action of tyrosinase on tyrosine. The first visible product is a red substance, which in later stages becomes reddish brown and finally black melanin. Thus, a second hypothesis, less likely than the foregoing, is that the melanoblast differentiates into only one kind of melanophore, which is more or less labile and synthesizes either phaeomelanin or melanin granules, depending upon external conditions.

The evidence seems clear that all the white breeds used in this study possess melanophores which are of a type peculiar to the donor. An *in vitro* study of skin-ectoderm explants from embryos of these breeds shows, in every case, the presence of melanophores containing melanin granules (Hamilton, unpublished results; see Dorris, 1936 and 1938, for their occurrence in the White Leghorn). Also, we have observed them directly in isolated pieces of the skin and feather germs (chiefly at their bases) of embryos of these breeds at stages just before hatching (also see Ris, 1940). Reasoning from analogy with the results obtained with the pigmented donors, it would appear that melanophores from the white-breed donors do enter and occupy definite positions in the feather germs of the pigmented host. Owing, however, to some peculiarity in their genetic constitution, few or no melanin granules are deposited, with the result that the host feather is white (Fig. 37). An examination of the feathers of these breeds reveals usually a complete absence of pigment granules (see Figs. 35–37). The melanophores of the White Leghorn are shown to differ from those of White Wyandotte, White Plymouth Rock, and White Silkie in being much less capable of producing an area of white feathers in pigmented hosts (see pp. 193–97 ff.).

From the analysis made above, it is concluded that the melanophore is a breed-specific cell whose action in a foreign feather germ is in accord with its genotypic composition. Furthermore, its action in controlling feather color pattern is, to a considerable degree, independent of the foreign-host environment. Similarly, Twitty (1936) and Twitty and Bodenstein (1939) in the studies on *Triturus* have shown that the pattern of melanophore distribution is determined primarily by species differences in the pigment-forming cells themselves.

The extent to which the melanophore behaves as an independent system in the production of the color pattern in the host feather remains to be considered. What factors peculiar to the host substratum, i.e., the epidermal collar of the feather germ, or humoral agents affect or modify the behavior of the melanophore? Important evidence on this question has come from a preliminary study of the patterns produced by melanophores derived from Barred Plymouth Rock, F_1-hybrid, and Robin donors. The pigment pattern formed as a result of barred implants exhibits considerable variation in width of the black and the white bars. In general, irrespective of the sex of the donor contributing the melanophores, these bars are wider in rapidly growing feathers, such as the wing primaries, and narrower in slower-growing feathers, such as the upper wing coverts and breast feathers. This is particularly convincing in those cases where variations in the width of the pattern occur on the same host, even though the melanophores all came from the same donor and the same region of its body. Also, in certain cases, i.e., Barred Plymouth Rock on New Hampshire Red host, or male F_1-hybrid on White

Leghorn host, some of the primary flight feathers show little or no white barring (trace on shaft only in some), whereas the late-emerging secondaries and upper wing coverts have a distinct barred pattern. It is thus apparent that rate of growth of the host feather germ is a factor in affecting barring pattern (cf. Montalenti, 1934). The production of a Robin-colored pattern in feathers of a White Leghorn host illustrates how the action of the Robin melanophore may be altered by the epidermal substratum in the feather germ of the host. In such feathers the apical end of the vane is reddish, and the remaining portion gray in color. For reasons similar to those given above in discussing the production of red-black color pattern in the New Hampshire Red, it is probable that these apicobasal differences in Robin coloration are under the control of regional differences in physiological activity within the host feather germ. The Robin melanoblast, under conditions peculiar to the apical portion of the feather germ, becomes a melanophore capable of producing red-pigment granules; whereas, in more proximal portions, it becomes a melanophore capable of forming black-pigment granules.

The distribution of the Robin-pigment granules in the down barbule cells of the host appears also to be under the control of the host epidermis. They are distributed more or less uniformly on the surface of the barbule cell, as is characteristic of fowls in general, and are not grouped at the nodes of the barbule, as occurs in the normal Robin (see Rawles, 1939, Figs. 9–13). A study of the feather germ for the origin of the structural differences found in the feather parts of the Robin and fowl may shed some light on how the distribution is effected. Differences in structural pattern within the feather germ may be of such a nature as to bring about a spacing or orientation of the melanophores and their processes peculiar to these two orders of birds, with the result that differences in pigment deposition and distribution to the epidermal cells occur.

It is well known from the work of Danforth (1933*a*), Lillie and Juhn (1932), and others, that humoral agents, such as thyroxine and sex hormones, may play a role in determining the kind of pigment pattern formed in the feather of adult plumage. The extent to which these agents modify or supplement the action of the melanophore in pattern production should be analyzed in the light of these experiments and conclusions.

Peculiar nature and behavior of the White Leghorn melanophore.—This investigation brings out clearly that the melanophores of the White Leghorn have a unique behavior in grafts. Implants containing White Leghorn melanophores produce an area of donor-colored feathers much less frequently and more limited in extent than do melanophores from any of the other white or any of the pigmented breeds tested. Implants to Black and Buff Minorca hosts give an area of white feathers with the highest frequency, which, however, is limited to the middle and distal portions of the wing. At first, implants of White Leghorn melanophores into Barred Plymouth Rock hosts yielded only negative results, but recently a few cases have been obtained in which a small area of white feathers did develop. So far, no positive results have been obtained on hosts of either New Hampshire Red or F_1-hybrid breeds. A similar peculiarity in the behavior of the White Leghorn melanophore in pigmented hosts has been noted by Dorris (1939), who found that they bring about the formation of white feathers on Australorp but fail to do so on Rhode Island Red hosts.

The question naturally arises as to the significance of this unique behavior of the White Leghorn melanophore when introduced into foreign hosts. Can it be correlated in any way with the peculiar genetic constitution of the White Leghorn race of fowls? This breed, according to the cross-breeding experiments of Hadley (1913, 1914, and

1915) and others, possesses (1) a white plumage character, which is dominant over dark color (for incomplete dominance over red and yellow see Danforth, 1933*b*) and (2) usually genic factors for black pigmentation and for barring. To explain the appearance of white in a bird with such genetic makeup, it has been generally assumed by poultry geneticists, following the suggestion of Hadley (1913), that an "inhibiting factor" is present which suppresses the formation of black pigment, not only in the feathers of the White Leghorn, which would be otherwise black, but also in the feathers, except for an occasional blackening, of crosses with black breeds. Beyond the conclusion that inherent black coloration is not expressed, no definite information is available concerning the nature of the mechanisms involved in this apparent inhibition.

Can this failure to produce black feathers be attributed to certain peculiar genetically controlled potentialities of the melanophores, or of the epidermal substratum, or to the combined action of these two components of a feather germ? The suggestion has been made that the epidermis contains a factor, chemical in nature, which prevents the melanophore from depositing pigment granules. The ability of the melanophore to produce pigment *in vitro* and *in vivo*, but inability to deposit pigment in the feather germ, appears to support this view. It has been observed that, when transplants are made to Leghorn hosts of older stages (i.e., at 5 days), the donor-colored area is generally smaller than in younger hosts. Does this indicate that the inhibitor factor is making or has made its appearance in the epidermis, as Dorris (1939) has suggested? The fact that an area of reduced dimensions is likewise produced, as a rule, when barred melanophores are grafted to New Hampshire Red hosts older than those usually employed (the number of negative cases also increases) makes this somewhat improbable. In all probability the formation of a smaller area in older hosts means that the conditions, whether mechanical or otherwise, suitable for the migration of the donor melanoblasts at the earlier stages are no longer favorable. Furthermore, the host melanoblasts have already migrated into the skin ectoderm before the donor ones are introduced; consequently, the latter have few or no available positions. Under such conditions the donor melanoblasts would be less effective in producing donor-colored feathers.

The strongest evidence against the view that the epidermis of the White Leghorn has a means for preventing pigment deposition is furnished by the general results of grafting. If an inhibitor factor were present in the epidermis, it would be expected that melanophores from pigmented donors would not be able either to produce or to deposit pigment granules in the White Leghorn feathers. Such is not the case, since all pigmented donors tested, very readily produced an area of pigmented feathers in the White Leghorn host. In no single case is the action of a melanophore from any pigmented breed of fowl, or even from a Robin, inhibited. It produces a color hue or a color pattern just as faithfully as it would in corresponding feather germs of donor control chicks.

Sooner or later, however, the donor pigment cells, irrespective of their source, apparently become exhausted, whereupon the feather becomes white or host colored. This usually takes place some time during the emergence of the juvenile plumage (see Fig. 20), but occasionally not until the adult plumage appears. Apparently, this does not indicate the onset of any new physiological conditions within the feather germs of the White Leghorn peculiar to it, since donor pigmentation becomes exhausted in an identical manner in hosts of all other breeds tested. In all probability, its disappearance is correlated with the quantity of melanophores originally introduced in the graft. If

implants containing a part of the neural crest where the potential melanophores are apparently concentrated are introduced, the effect may persist even into the adult plumage—many months after the operation (as long as 18 months in one case). With implants containing less melanophores, i.e., head skin ectoderm (see p. 182), the effect may persist only during the formation of the apical ends of the juvenile contour feathers.

In this connection it is important to inquire why, in general, the donor melanophores fail to persist throughout the life of the host, especially since there is no apparent incompatibility between host and donor melanophores. Obviously, the melanophores are being used up continuously in the growing feather of the host, and new ones must be forthcoming from some source as the new feather germ organizes, following each molt. A superfluous supply should be available, since no host melanophores were removed at the time of grafting. It is probable that a special zone of the follicular epithelium, described by Lillie and Juhn (1938) as "regenerating cells," is the source of the reserve melanophores. A special study of this zone in a regenerating feather may shed some light not only on their source but also on why the melanophores of the donor are sooner or later replaced by those of the host. It is, of course, conceivable that long-continued residence in a foreign host may alter the melanophores physiologically, so that ultimately they are more easily supplanted by those of host origin, or that an immunity reaction is built up against them.

This replacement of the donor with host melanophores in these experiments is in marked contrast to results obtained by Danforth (1937 and 1939) in grafts of skin made after hatching. He noted only a slight tendency for the melanophores of the grafted skin to be replaced by those of the host, even after several natural and induced molts. Conditions fundamentally different in the two methods of grafting may account for this. When the transplantation is made early in embryonic life, the only foreign element in the host skin is the donor melanophore; whereas, after hatching, the donor furnishes the dermis and the epidermis, as well as the melanophores. When this difference is considered, the results are much less surprising.

Having argued, from the available evidence, that the epidermis of the White Leghorn has no properties essentially different from those of other breeds which could explain the lack of pigmentation, we shall now turn to the melanophores themselves for a possible clue. It was first thought that their ability to produce white feathers in Buff and Black Minorca hosts could be attributed to some constitutional properties possessed by these hosts and not by any of the others tested. This seemed particularly convincing when it was noted that those hosts giving positive effects are rapid feathering, while those giving negative results are slower-feathering races. Since, however, the white-feathered area was smaller in the Minorca hosts, much in contrast to the large areas produced by White Silkie implants in hosts of the same breed, it next occurred to us that these results might be explained, at least in part, on the hypothesis that the melanophores of the White Leghorn (*a*) are quantitatively fewer or (*b*) have less ability to migrate than in other breeds. In a preliminary study of this matter it has been found that pieces of skin ectoderm, including subjacent cells of the neural crest from the White Leghorn, will occasionally produce a definite area of white feathers on a Barred Plymouth Rock chick, a host heretofore always negative (see Fig. 11). The white area is of limited dimensions, however.

Apparently only when large numbers of potential melanophores are introduced can the donor effect be produced in the Barred Plymouth Rock host, and even then it is limited

in its extent. It cannot be decided from this result alone whether the donor melanophores are merely few in number or somehow limited in their powers to migrate, or whether both conditions are concerned. Observations on living feather germs of the White Leghorn indicate that the number of melanophores containing black pigment may be relatively fewer than in other breeds (Ris and Hamilton). Also, their number apparently varies greatly from individual to individual. On the basis of a limited migratory power, especially of a retarded rate of spread, the host melanophores would be expected to reach the host epidermis before the donor ones do, with the result that no donor effect is produced. This could explain the absence of the donor effect commonly found. A still more puzzling problem is why they apparently reach their positions in the epidermis more readily in a fast-feathering, than in a slow-feathering, host. This matter of the rate and extent of spreading of the melanophores of the White Leghorn breed—and, indeed, of all breeds—furnishes a problem for further investigation.

The restricted power of the White Leghorn melanophores to produce an area of white feathers in pigmented hosts, whether due to limited numbers or to some weakness in migration tendency, points to the interpretation that the melanophores themselves are responsible for the peculiarity in behavior noted. This viewpoint is strongly supported by the demonstration that the Leghorn feather germ exhibits no tendency whatsoever to prevent melanophores of colored breeds from forming and depositing pigment in it. Moreover, from an analogy with the action of melanophores of other breeds, where each acts primarily as an independent system in producing color or color pattern, it is reasonable to conclude that this peculiarity must be attributed largely to the action of the White Leghorn melanophore itself. Like all the other melanophores examined, its genetic constitution apparently fixes its ability to act and react in the foreign host environment.

Another striking characteristic of the White Leghorn melanophore is its limited capacity to deposit pigment. An examination of a White Leghorn feather germ shows the presence in the epidermal collar of normally branched melanophores containing black-pigment granules. As the parts of the feather arise from the collar, the melanophores, as a rule, withdraw their processes, round up, and die. The result is that few or no pigment granules are deposited in the epidermal cells of the feather parts. Apparently the melanophores of this breed usually have, at first, the capacity to synthesize black melanin pigment; but, as they grow older, some change of a lethal nature takes place which prevents them from forming additional pigment or from depositing that already formed.

This peculiar behavior is apparently an intrinsic property of the melanophore. This is brought out very nicely in a study of its action in the Minorca and Barred Plymouth Rock hosts. In the feather germs of these hosts, where normally pigment granules are readily deposited, it can produce white feathers. Even under conditions favorable for pigment synthesis and deposition, it still lacks the ability to deposit pigment. Apparently, these pigmented hosts do not supply to the White Leghorn melanophore the factor necessary for pigment formation and deposition.

In the White Leghorn itself, the ability to deposit pigment in the feathers is apparently not controlled by the epidermal substratum, since, as noted above, the latter does not hinder the synthesis and deposition of pigment granules on the feathers by melanophores of any breed thus far grafted. The melanophore thus appears to be quite

independent of the epidermal substratum. This furnishes additional support for the view that the limitation of melanin deposition is primarily under endogenous control.

The extent to which the melanophore of this breed is independent of exogenous conditions is a question of some significance. Such external factors as enzymes, humoral agents, and the like must be recognized as playing a possible role in modifying the expression of some of the genetic potentialities of these cells.

SUMMARY

1. This report deals with the control of feather color pattern by grafting a small piece of skin ectoderm or pure mesoderm from one embryo to another of the same age (67–108 hours' incubation) but of a genetically different breed. The graft site was usually the base of the right wing bud; but other regions, such as the leg and tail buds and the head, were sometimes used.

2. Isolates from the head or trunk regions, at all ages used, usually produce an area of donor-colored down feathers on the host at and about the site of grafting, often extending to adjacent regions. Skin ectoderm or mesoderm from the wing bud of embryos younger than 80 hours or from the leg bud earlier than 96 hours fails to produce such an area.

3. An area of donor-colored feathers has been obtained in all host-graft combinations tested between two pigmented and between pigmented and unpigmented breeds. The White Leghorn, in contrast to all other donor breeds, produces a very restricted area of donor-colored feathers and with a lower frequency.

4. The donor-colored down feathers are gradually and normally replaced by contour feathers of the junvenile plumage having the color or color pattern of the donor breed; but as regards shape, size, and distribution in tracts, they are identical with corresponding host control feathers.

5. The donor-colored plumage is ultimately replaced by host-colored feathers. If this occurs during the emergence of the juvenile or adult feather, it becomes a mosaic of donor and host coloration. Usually the donor effect disappears with the molting of the juvenile plumage, but occasionally it persists in some of the feathers of the first set of adult plumage. The time of disappearance of the donor coloration seems to be roughly proportional to the quantity of melanophores brought in by the implant.

6. The feathers of the donor-colored area are structurally of host epidermal origin. This is shown by (*a*) the failure of the skin-ectoderm implant to replace the host epidermis, (*b*) the production of donor-colored feathers with grafts of pure mesoderm, (*c*) the development of structurally normal (non-Silkie) contour feathers in Black Minorca or Barred Plymouth Rock hosts following the implantation of Silkie skin ectoderm, and (*d*) the formation of Robin-colored contour feathers of typical fowl structure by grafting Robin-skin ectoderm to the White Leghorn.

7. The feather coloration is controlled through the agency of melanophores, which migrate from the implant into the host feather germ, there depositing specific melanin granules of the donor type in the epidermal cells of the developing feather.

8. Evidence is presented to show that melanophores migrate shortly after 80 hours into the wing, and after 96 hours into the leg, from an outside source.

9. The neural crest as the exclusive source of melanophores is discussed. In presomite blastoderms the capacity to form melanophores coincides with the neural-forming area.

10. The action of the melanophore in controlling color or color pattern in the host feather is in accord with its genotypic constitution, i.e., faithful production of donor color pattern and deposition of donor-specific melanin granules in the host feather and is highly independent of the foreign host environment.

11. Melanophores from the white breeds apparently enter and occupy definite positions in the feather germs of colored host breeds. Because of some peculiarity in genetic constitution, they deposit few or no melanin granules, with the result that the host feather is white.

12. The peculiar behavior of the White Leghorn melanophores in grafts is an intrinsic property since (1) the epidermis of a pigmented breed does not change their limited power to synthesize and deposit melanin and (2) the epidermis of the White Leghorn never inhibits melanin synthesis and deposition by melanophores from pigmented breeds.

13. Finally, the extent to which the melanophore behaves as an independent system in the production of color pattern in the host is considered. Extrinsic factors (e.g., growth-rate of feather germ, humoral agents, etc.) are shown to play a role.

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PLATE I

FIG. 2.—Photograph of a living 72-hour host chick embryo showing a piece of stained skin ectoderm grafted to the base of the right wing bud. × *ca.* 5.

FIG. 3.—A 5-day-old White Leghorn chick exhibiting an area of black down on top of the head. Produced by grafting to the hindbrain region of the host embryo at 79 hours a piece of skin ectoderm from the base of a wing bud of a Barred Plymouth Rock donor embryo of the same age.

FIG. 4.—A 22-day-old New Hampshire Red chick showing barred plumage on the right wing. Produced by grafting to the wing-bud base of the host embryo at 82 hours a piece of head skin ectoderm from a Barred Plymouth Rock embryo of the same age.

PLATE II

FIG. 5.—A 1-day-old ♂ White Leghorn chick showing area of black down feathers (first few primary flight feathers partly emerged) covering entire wing and part of breast. Produced by grafting to wing-bud base of the host embryo at 78 hours a piece of head skin ectoderm from a Barred Plymouth Rock embryo of the same age.

FIG. 6.—Same chick as the one shown in Fig. 5, 18 days after hatching.

FIG. 7.—Same chick as those shown in Figs. 5 and 6, 31 days after hatching. Note barred pattern in contour feathers of wing and breast.

PLATE III

FIG. 8.—A 4-day-old Black Minorca chick showing an area of white plumage covering the entire wing and right side of breast. Produced by implanting into the base of the wing bud of the host embryo at 74 hours a piece of head skin ectoderm from a White Wyandotte embryo of the same age.

FIG. 9.—A 23-day-old Barred Plymouth Rock chick showing white plumage on the right wing. Produced by grafting to the wing-bud base of the host embryo at 70 hours a piece of head skin ectoderm obtained from a White Silkie bantam embryo of 71 hours' incubation.

PLATE IV

FIG. 10.—A 34-day-old ♀ White Leghorn chick showing solid black contour feathers on the right wing. Produced by grafting to the wing-bud base of the host embryo at 73 hours a piece of head skin ectoderm from a Black Minorca embryo of the same age.

FIG. 11.—Tip of the eighth primary and six upper coverts of a 1-month-old Barred Plymouth Rock chick showing solid white coloration. Produced by transplanting to the wing-bud base of the host embryo at 78 hours the neural crest region (including upper part of neural tube and somite and overlying skin ectoderm from posterior level of body) of a White Leghorn embryo of the same age.

PLATE V

All feathers shown are remiges of the juvenile plumage of the host chick.

FIG. 12.—Tenth secondary of a White Leghorn host showing a barred pattern produced by melanophores from a Barred Plymouth Rock implant (from same chick shown in Fig. 7).

FIG. 13.—Seventh primary of a New Hampshire Red host showing a barred pattern produced by melanophores from a Barred Plymouth Rock donor.

FIG. 14.—Seventh primary of a White Leghorn host showing a red-and-black (to left of shaft) pattern produced by melanophores derived from a New Hampshire Red implant.

FIG. 15.—Eleventh secondary of a White Leghorn host showing solid red coloration produced by melanophores from a Buff Minorca implant.

PLATE I

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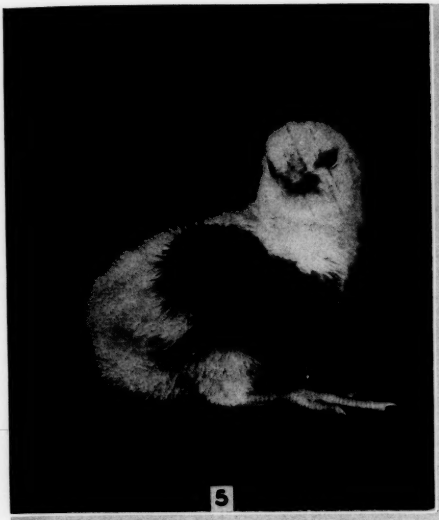
PLATE II



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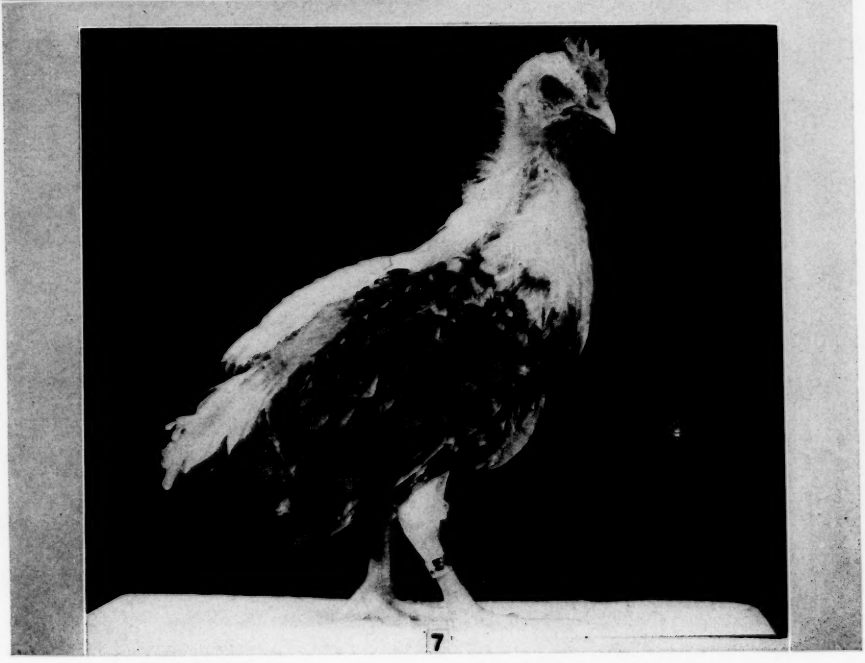

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PLATE III

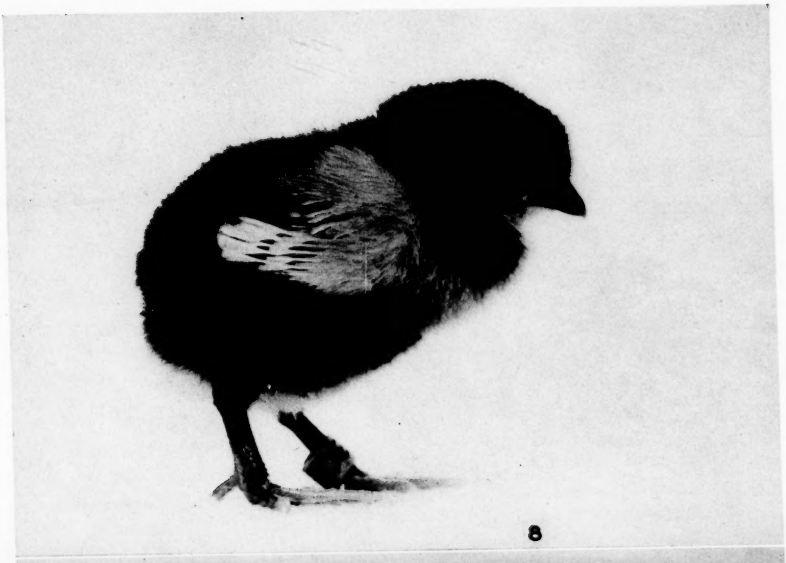

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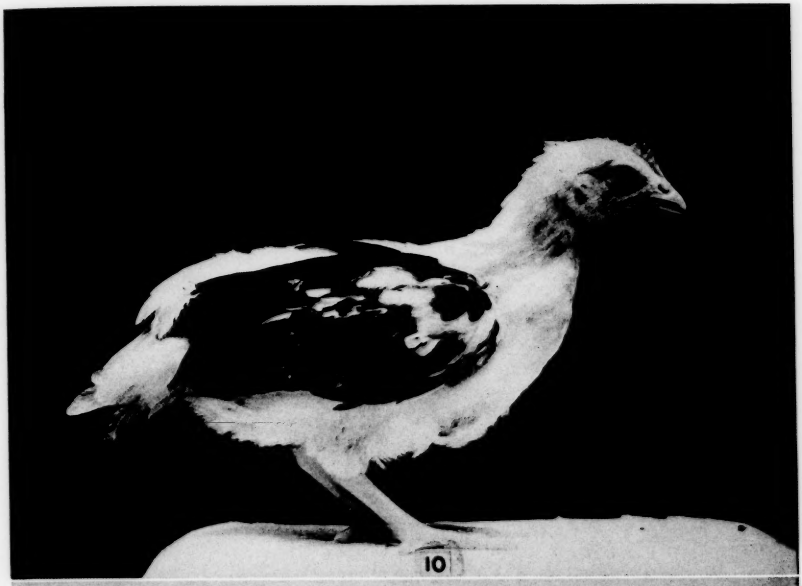
PLATE IV



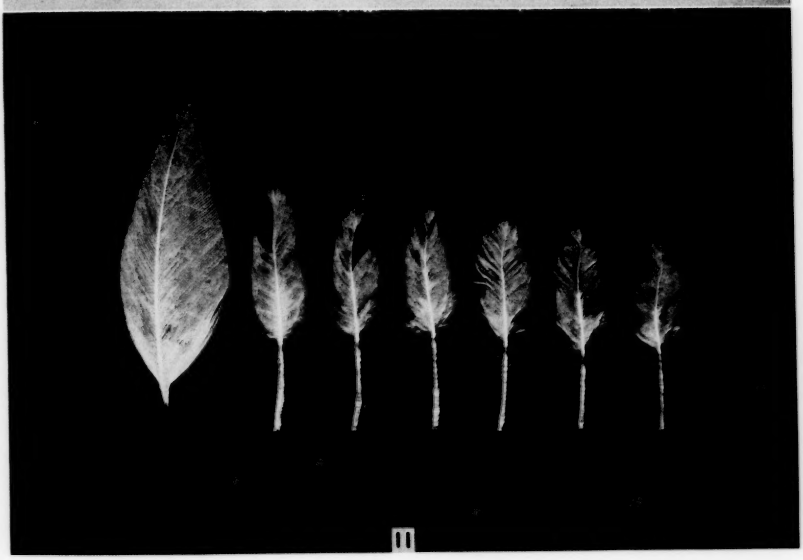


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PLATE V

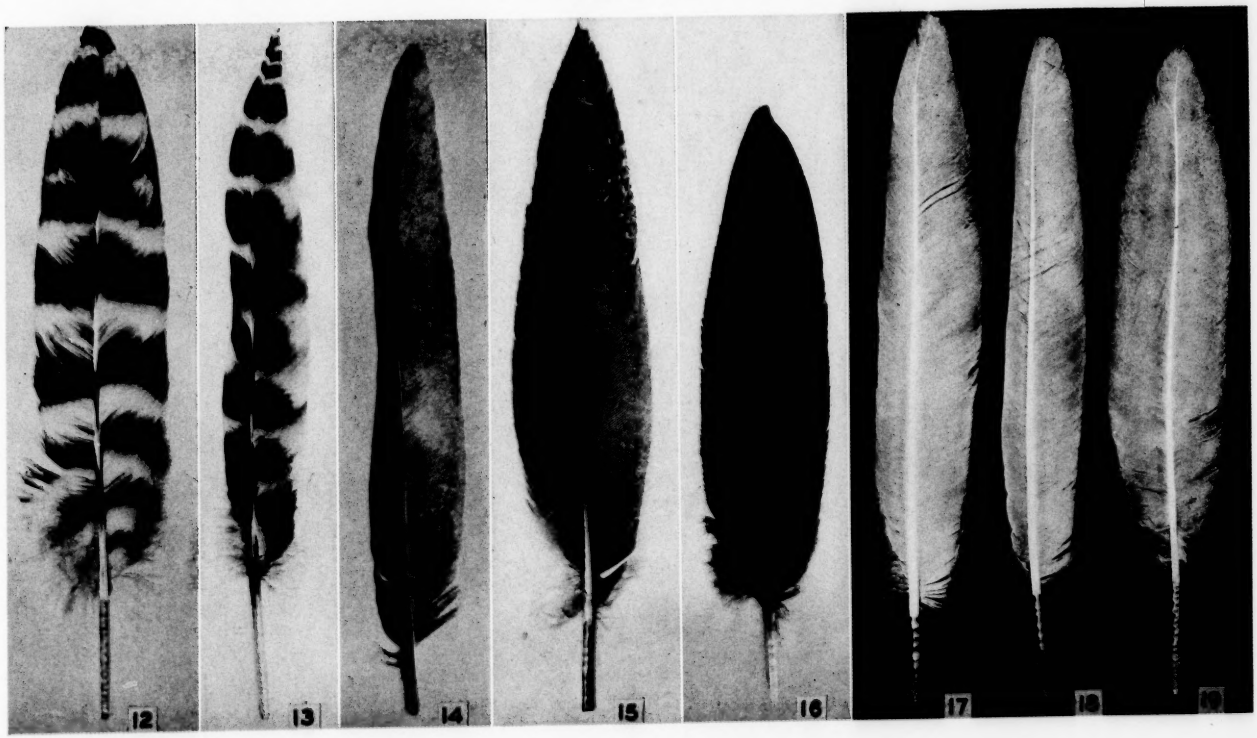

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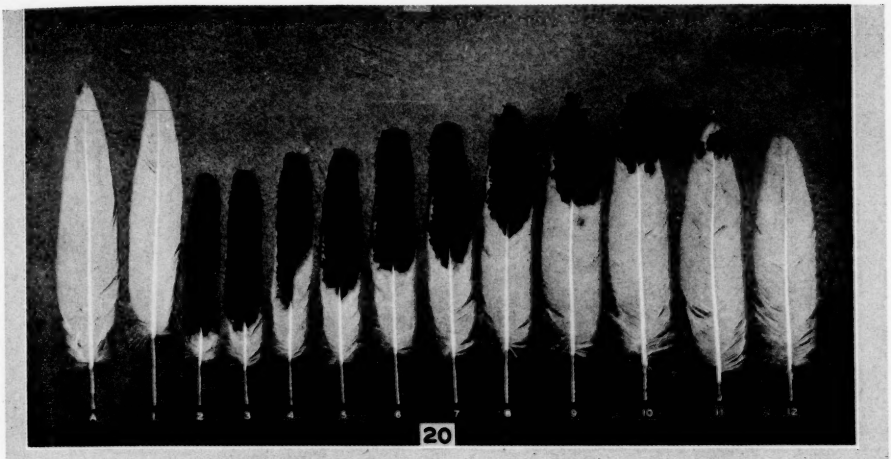
PLATE VI



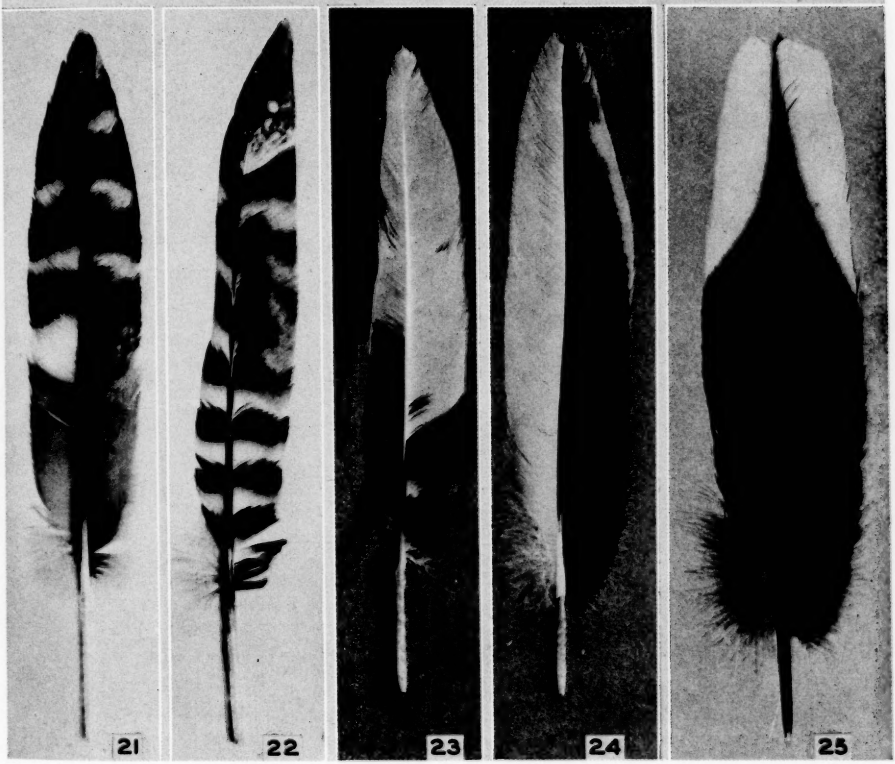


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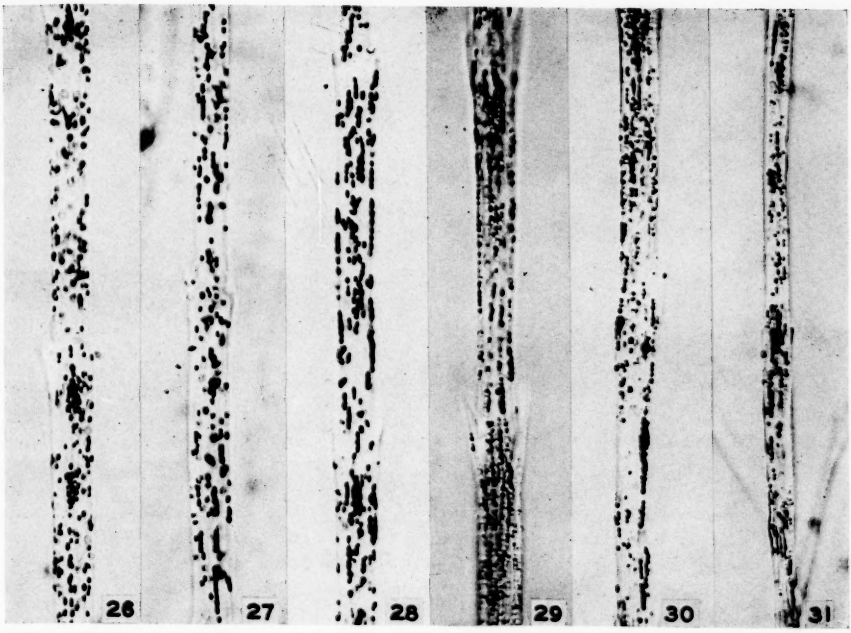
PLATE VII



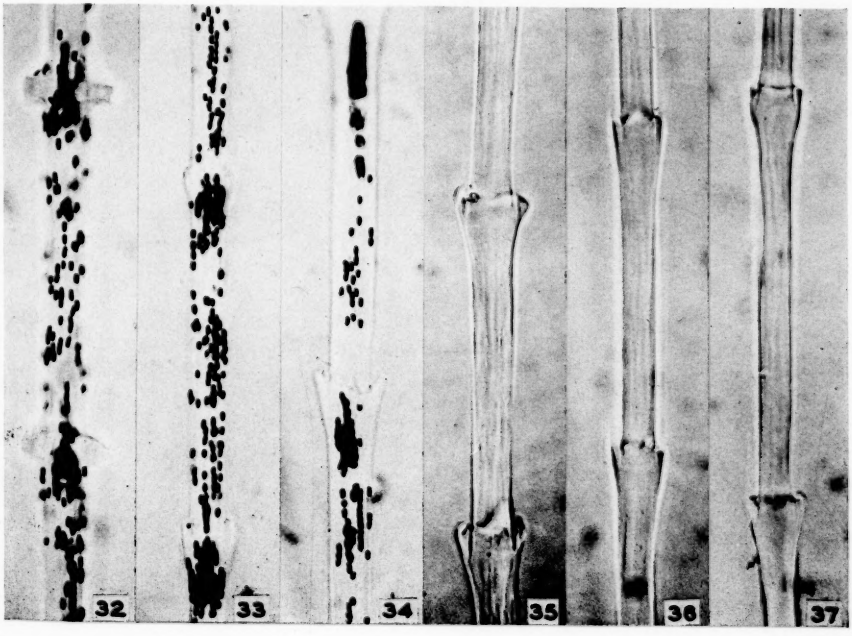


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FIG. 16.—Axial feather of a Barred Plymouth Rock host showing solid black coloration produced by melanophores from a Black Minorca implant.

FIG. 17.—Second primary of a Barred Plymouth Rock host showing solid white coloration produced by melanophores from a White Wyandotte implant.

FIG. 18.—Fifth primary of a Black Minorca host showing solid white coloration produced by melanophores from a White Silkie implant.

FIG. 19.—Third secondary of a Barred Plymouth Rock host showing solid white coloration produced by melanophores from a White Silkie implant.

PLATE VI

All feathers, except as noted, are remiges from the juvenile plumage of the host chick.

FIG. 20.—Axial (*A*) and secondary (1–12) feathers of a White Leghorn chick which received at 74 hours' incubation an implant of head skin ectoderm from an F_1 hybrid ♀ donor. The extent of hybrid pigmentation produced in a feather varies directly with the time of its emergence, being greatest in the first feather (No. 2) and least in the last ones to emerge. The time of disappearance of pigmentation in the various feathers is approximately simultaneous.

FIG. 21.—Seventh secondary from a New Hampshire Red host. The apical two-thirds has the barred pattern of the Barred Plymouth Rock donor; and the proximal third, the black-and-red pattern of the host.

FIG. 22.—Seventh primary from a Barred Plymouth Rock host. The apical one-fourth has the black-and-red pattern of the New Hampshire Red donor; and the proximal three-fourths, the barred pattern of the host.

FIG. 23.—Third primary from a Barred Plymouth Rock host. The apical half is white, like the White Wyandotte donor; and the proximal half has the barred pattern of the host.

FIG. 24.—Third secondary of a Black Minorca host. The left half of the vane is white, like the White Silkie donor; and the right half is mostly black, like the host.

FIG. 25.—Tenth secondary of the first adult plumage of a White Leghorn host. Outer portions of the vane at the tip are white, like the host; the remainder, solid black, like the Black Minorca donor.

PLATE VII

Photomicrographs of unstained whole mounts of barbules from the downy region of covert and flight contour feathers, with the exception of Fig. 31, which comes from the down plumage. × *ca.* 810.

FIG. 26.—Melanin granules of Barred Plymouth Rock control.

FIG. 27.—Melanin granules of Barred Plymouth Rock deposited in White Leghorn.

FIG. 28.—Melanin granules of Barred Plymouth Rock deposited in New Hampshire Red.

FIG. 29.—Melanin granules of New Hampshire Red control.

FIG. 30.—Melanin granules of a New Hampshire Red deposited in White Leghorn.

FIG. 31.—Melanin granules of a New Hampshire Red deposited in Barred Plymouth Rock.

FIG. 32.—Melanin granules of Black Minorca control.

FIG. 33.—Melanin granules of Black Minorca in White Leghorn.

FIG. 34.—Melanin granules of Black Minorca in Barred Plymouth Rock.

FIG. 35.—White Leghorn control showing absence of pigment granules.

FIG. 36.—White Silkie Bantam control showing absence of pigment granules.

FIG. 37.—Black Minorca barbule showing absence of pigment granules when White Silkie is the donor.

THE LOCALIZATION OF PIGMENT-FORMING AREAS IN THE CHICK BLASTODERM AT THE PRIMITIVE-STREAK STAGE[1]

(Three text figures and one plate)

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RECENTLY a number of investigators have attacked problems relating to the production of pigment by embryonic chicks. In 1937 Willier, Rawles, and Hadorn made skin grafts between different breeds of chick embryos and found that a piece of ectoderm from the head of a potentially colored embryo had the capacity to produce a pigmented area when grafted to the wing bud of a noncolored host. In a later contribution Willier and Rawles (1938*b*) reported that a piece of limb-bud mesoderm from a colored donor would also produce the same result.

Dorris (1936, 1938*a*, 1938*b*, 1939) was able to show that neural crest cells from a colored embryo produced melanin not only when transplanted to the limb bud of a colorless host but also when cultivated *in vitro*.

Eastlick (1938) corroborated Dorris' conclusion that pigment cells are derived from the neural crest by making reciprocal limb-bud transplants between embryos of pigmented and nonpigmented breeds. He found that the pigment-forming cells of 56–72-hour Brown Leghorn or Barred Plymouth Rock embryos were concentrated along the neural tube. By varying the position of the cut made in removing the limb-bud primordium of a potentially colored embryo, it was possible to secure a nonpigmented, a partly pigmented, or a fully pigmented graft when the bud was transplanted to a White Leghorn host. These results indicated that the pigment-producing cells migrate peripherally from the neural tube and later occur in the ectoderm and mesoderm.

The present study was made in an attempt to gain information concerning the localization of pigment-forming areas in pre-somite chick embryos. Accurately measured portions of blastoderms of Barred Plymouth Rock embryos in definitive primitive streak stages (13–20 hours of incubation) were transplanted to White Leghorn hosts which possessed between twenty-six and thirty-three somites. The presence or absence of pigmented feathers on the host was a criterion of whether the grafted area included cells which were able to differentiate into melanophores.

A demonstration of this work was given at the spring meeting of the American Association of Anatomists in Boston (Eastlick, 1939*a*). At that time Rawles and Willier (1939) reported a paper on a similar subject. While the blastoderms were sectioned at different levels in the two laboratories, and slightly different procedures were followed in grafting the pieces to the hosts, the results seem to supplement each other and appear to warrant the same general conclusions.

EXPERIMENTAL PROCEDURES

The following procedure was used in making the operations. The egg which contained the donor embryo was opened after sterilizing the shell with alcohol, and the

[1] This investigation was aided by a grant from the Research Council of the University of Missouri.

region of the primitive streak was stained by means of an agar block which contained neutral red. The blastoderm was then severed from the yolk, removed to warm, sterile salt solution, and washed free of adhering yolk granules. The blastoderm was flattened by pipetting away most of the fluid and then examined under a dissecting microscope.

TABLE 1

SUMMARY OF RESULTS OBTAINED WHEN VARIOUS PIECES OF PRIMITIVE STREAK BLASTODERMS OF BARRED PLYMOUTH ROCK EMBRYOS WERE GRAFTED TO THE LATERAL BODY WALL OF WHITE LEGHORN EMBRYOS

SERIES A

	Piece Grafted (See Fig. 1)			
	1	2	3	4
Pigment produced	3	18	0	0
No pigment produced	20	5	19	13
Died before ninth day	9	12	12	10
Total	32	35	31	23

SERIES B

	Piece Grafted (See Fig. 2)				
	A	B	C	D	E
Pigment produced	2	12	13	0	0
No pigment produced	15	5	5	17	14
Died before ninth day	8	10	5	7	9
Total	25	27	23	24	23

SERIES C

	Piece Grafted (See Fig. 3)										
	1	2	3	4	5	6	7	8	9	10	11
Pigment produced	1	2	0	3	8	1	2	9	1	0	0
No pigment produced	11	10	9	10	1	10	6	2	13	10	7
Died before ninth day	5	6	7	5	7	7	9	7	5	8	5
Total	17	18	16	18	16	18	17	18	19	18	12

Measurements of the length of the streak, the distance from the primitive pit to the anterior edge of the area pellucida, and the width of the area pellucida at the pit level were made by means of an ocular micrometer. An outline of pieces of the desired size was made by means of a glass needle, according to the method described by Rawles (1936).

In preparing the host, an opening 5–7 mm. square was made in the shell directly over the embryo. The shell membranes were removed in the region of the opening, and the body wall of the embryo was stained with an agar block saturated with neutral red. The vitelline membrane was slit, and the amnion torn open whenever necessary. A small incision was made in the body wall just posterior to the wing bud and slightly lateral to the posterior cardinal vein. A full description of this procedure has been given by Hamburger (1933, 1938).

The position of the cuts that were made in separating the blastoderm into pieces may be ascertained by referring to Figures 1–3. In all cases an attempt was made to remove the mesentoderm from the mesectoderm. The removal was made quite easily in the pieces anterior to the node; but in the region of the streak, the close adherence of the layers usually prevented their complete separation.

The desired piece of blastoderm was drawn up with a Spemann pipette and transferred to the lateral body wall of the host. It was oriented within the incision by means of a glass needle. As the wound healed, the piece of blastoderm became incorporated in the body wall of the host. In some cases the transplanted portions were pushed through the incision into the intra-embryonic coelom and oriented over the vitelline artery rather than being left within the slit in the body wall.

All hosts were allowed to develop 15 or more days. The majority were sacrificed between 17 and 19 days, while a few were allowed to hatch.

OBSERVATIONS

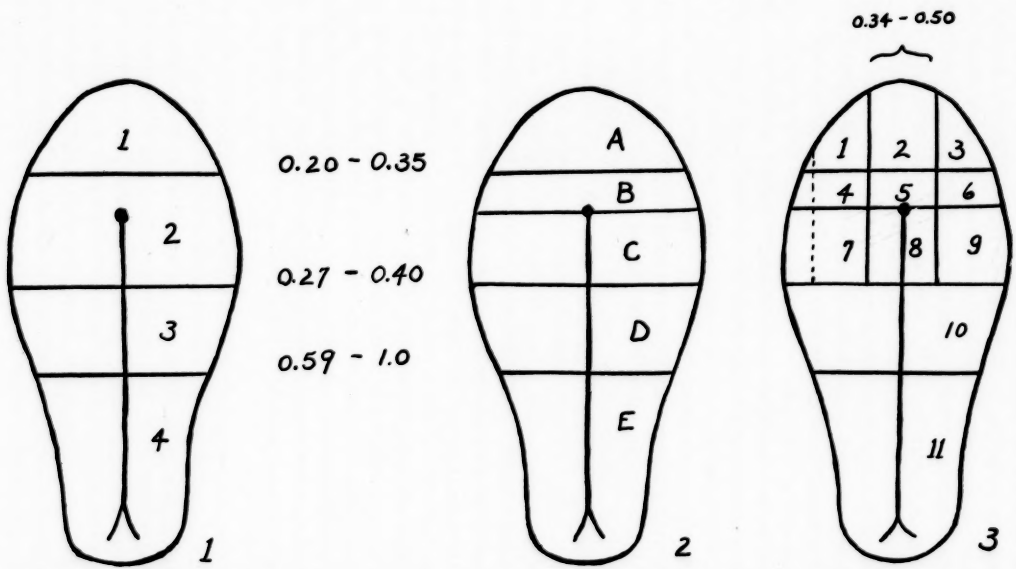
Series A.—Table 1 presents the summary of the results which have been obtained in the three series of experiments. In the table the grafted pieces are designated in the same manner as in Figures 1–3. The measured limits of the grafted pieces may be noted by referring to the diagrams.

The most anterior piece in Series A developed pigment in approximately 13 per cent of the cases (see Table 1, Ser. A). Even in the positive cases the pigmented areas were small and possessed feathers which were only partially pigmented. In some of these mosaics a portion of the feather was colored, and the rest of it was nonpigmented; while in others the pigment was laid down irregularly throughout the whole feather, so that a streaked appearance resulted.

Piece 2 in Series A developed pigment in 18 out of 23 surviving cases, or in approximately 78 per cent of the grafts. A large area of pigmented feathers usually occurred in these hosts. In one case the entire lateral body wall, the wing, and the breast of the host became intensely pigmented. The melanophores migrated from the point of the incision and invaded a large surrounding area. Moreover, a few of the transplants which were made into the intra-embryonic coelom developed into good-sized masses which bore colored plumage (Fig. 4).

An examination of the positive cases reveals that variations occur in the size of the colored patches and in the degree of coloration of the feathers. This may be associated with the degree of incorporation of the graft, its viability, etc. It is significant, however, that the portion of the blastoderm which contained the node invariably produced a higher percentage of positive cases than other regions.

Series B.—The second set of experiments was designed to test whether a transverse strip of blastoderm containing the anterior half of the node has greater capacity to produce melanophores than a piece containing the posterior half of the node. The re-



FIGS. 1–3.—Diagrams which illustrate the location of cuts made in the blastoderm. The measurements (in millimeters) represent the distance from the primitive pit. Blastoderms sectioned as in Fig. 1 constitute Series A; as in Fig. 2, Series B; etc.

sults which have been obtained are inconclusive, since the number of successful cases is approximately equal in both cases (see Table 1, Ser. B). Both anterior and posterior pieces produced pigment in approximately 70 per cent of the hosts which lived long enough to allow one to make certain of the presence of colored feathers. An examination of the hosts has failed to reveal any significant differences either in the intensity of the pigmentation or in the extent of the area which is colored (Figs. 5 and 6).

The results which have been obtained by grafting the anterior piece *A* and the posterior pieces *D* and *E* are strictly comparable to those obtained in Series A. Two positive cases only were secured from grafts of piece *A*. In each of these a small area was pigmented, and many of the feathers were mosaics. The posterior pieces gave negative results in all cases.

Series C.—This series was made in an endeavor to locate within rather close limits the regions of the blastoderm which differentiate pigment cells. A summary of the results obtained in these experiments is shown in Table 1, Series C. Reference to the table reveals that the pieces which are cut 0.2–0.35 mm. anterior to the node have a restricted pigment-forming capacity. While the number of experiments is not large, the results suggest that the left anterior piece 1 has a higher potency than the right anterior piece 3 and that the middle section has the greatest potency of all. Even in the positive cases the pigmented area is small (Fig. 7), and the feathers are partly colored.

Pieces 4, 5, and 6 are strips, 0.2–0.35 mm. in width, which extend anteriorly from the mid-portion of the node. Each of these pieces has the capacity to differentiate pigment cells, but the number produced by the three pieces varies greatly (see Table 1). The left lateral piece 4 produced colored patches of feathers in 23 per cent of the cases. In two of the three positive cases a small area of colored down was produced; in the other, a fairly extensive pigmented area occurred. The center piece 5 which included the anterior half of the node, produced pigment in approximately 90 per cent of the cases. Not only was a higher percentage of positive cases secured from this piece than from any other portion of the blastoderm, but a larger pigmented area usually resulted. Moreover, most of the feathers are intensely pigmented. Only a single positive case has been secured from the right lateral piece 6. A small number of pigmented feathers occurred on this host (Fig. 8).

These results suggest that the left lateral pieces have a greater potency than the right lateral portions of the blastoderm. This same result was procured in pieces from the next level, namely, pieces 7, 8, and 9. The lateral pieces 7 and 9 produced approximately the same percentage of positive cases as pieces 4 and 6. Moreover, the extent of the pigmented areas is approximately the same in these pieces. Piece 8, which contained the posterior portion of the node, was able to develop pigment in approximately 80 per cent of the cases. No significant differences were noted in the intensity of the coloration or in the size of the pigmented areas produced by the anterior and posterior portions of the node. While the former produced a slightly higher percentage of positive cases, the difference does not seem to be statistically significant. The results show, beyond doubt, that the node produced more melanophores than other regions of the blastoderm.

As in Series A and B, the two posterior pieces failed to produce pigmented feathers. In one case a nodule which possessed noncolored feathers was formed on the lateral body wall at the point of the incision.

Forty-one operations were made in which pieces of blastoderm, sectioned as in Figure 3, were placed into the intra-embryonic coelom of the hosts. Of the four positive cases secured, three were transplants of piece 5, the other of piece 8. The blastoderm is very fragile, and apparently the majority of pieces disintegrate before receiving an adequate blood supply. Since such a small number of positive cases was secured, this method was discontinued.

So far, the earliest stages found to produce pigment were transplants of the middle third of 8-hour blastoderms. Two cases out of 12 were positive. A small patch of pigmented feathers resulted in each case. These results, while preliminary in nature, suggest that the blastoderm earlier than the definitive stage has relatively restricted pigment-forming capacity. It seems probable, however, that positive cases can be secured from all stages, including the unincubated blastoderm, since Butler (1935) has obtained nervous tissue in pieces of unincubated blastoderm transplanted to the chorioallantoic membrane, and Dalton (1935) has found medullary tissue in transplants of pieces of 8-hour blastoderms. A much larger number of experiments, however, will have to be made before any general conclusions can be drawn concerning the capacity of these early stages to develop pigment.

DISCUSSION

The foregoing experiments demonstrate that various regions of the blastoderm of Barred Plymouth Rock embryos at definitive primitive streak stages possess striking differences in their capacity to produce melanophores. Hensen's node seems to have the greatest ability to produce pigment. This is shown by the fact that pieces of blastoderm containing the node produce more positive cases than fragments from other regions. Moreover, an extensive heavily pigmented area usually results from such grafts. The pigment cells spread from the point of the incision and feed melanin into the developing follicle cells of the host.

When the node was bisected transversely, no significant differences were found in the pigment-forming capacity of the two halves (Figs. 5 and 6). The results are not strictly comparable, since the anterior was smaller than the posterior piece. Probably the position assumed by the blastoderm within the incision is of greater significance than the size of the grafted fragment. For example, the smaller piece may be placed in the slit in such a way that a greater percentage of its cells becomes incorporated in host tissue than may be the case with the larger strip. In the latter instance a considerable portion of the transplant may disintegrate, and only those cells develop which heal into the wound. Some of the most pronounced cases of pigmentation were the result of the transplantation of the smallest piece (5 in Ser. C).

The lateral pieces of the blastoderm 4 and 6, 7 and 9, have the capacity to produce melanophores, but the potency is much more restricted than in the region of the node. Probably the majority of melanophores arising from these pieces were developed in those portions which lie nearest the node. Twelve experiments have been made in which pieces of the blastoderm, cut 0.5–0.6 mm. to the left of the pit (regions lateral to the broken line in Fig. 3), were transplanted to the body wall of embryonic hosts. Negative results were secured in all cases.

Rawles (1936), in a study of the potencies of pieces of blastoderm as revealed by transplantation to the chorioallantoic membrane, found that the greatest number of positive cases were secured in fragments which included the node or were directly ad-

jacent to it, and that the percentage of "takes" diminished with increasing distance from this point. It is possible, therefore, that the ability of the different pieces of blastoderm to produce pigment may be associated with their capacity to become incorporated and to survive and grow. For example, the small number of pigmented cases secured after transplantation of the most anterior or lateral pieces may be due to the poor survival of these fragments rather than to their inherent inability to produce pigment. While this possibility cannot be excluded, the fact that some of these fragments gave rise to nodules which bore noncolored plumage is opposed to such an interpretation.

The localization of the pigment-forming areas noted in this study is essentially the same as that shown by the researches of Rawles and Willier (1939). They sectioned the blastoderm at levels slightly different from those used in this study; but, in general, very close agreement seems to have been reached concerning the localization of the melanophore-forming areas at the primitive-streak stages.

Investigations of the organization of the primitive-streak blastoderm of chick embryos have been made by injuring or vital-staining localized areas, by explanting pieces to the chorioallantoic membrane, or by culturing them *in vitro*. The results secured by these various methods have indicated that Hensen's node and the regions immediately surrounding it have the greatest capacity to produce medullary tissue. The various workers, however, are not in entire agreement regarding the exact limits of the area concerned.

Hunt (1932) reported that pieces of blastoderm cut more than 0.3 mm. anterior to the node possessed little capacity to develop neural tissue when transplanted to the chorioallantoic membrane. Rudnick (1938*a*), using tissue-culture methods, has substantiated these results. Apparently, the lateral limits of the area which produces the medullary plate have not been investigated extensively. Hunt (1932) reported that medullary tissue developed in very few transplants of pieces of blastoderm cut more than 0.3 mm. lateral to the pit. The studies of Wetzel (1929) and of Pasteels (1936) seem to substantiate this conclusion. A number of reports have appeared concerning just how far posterior to the pit neural tissue may be differentiated. Hunt (1931, 1932), using the chorioallantoic technique, reported that he was unable to secure central nervous tissue from pieces of the blastoderm cut more than 0.21 mm. posterior to the pit. Wetzel (1929) and Pasteels (1936) found that the ectoderm as far back as one-half the distance from the pit to the posterior end of the streak contributed to the formation of neural tissue.

Dalton (1935), by the use of chorioallantoic grafts, and Waddington (1935), by *in vitro* methods, showed that pieces of blastoderm at least 0.6 mm. posterior to the pit formed medullary plate. Rudnick (1938*a*, 1938*b*) cultured carefully measured pieces of the blastoderm and found that pieces more than 0.4 mm. posterior to the pit failed to develop central nervous tissue.

The foregoing data seem to indicate that an area extending 0.3 mm. anterior to the pit, 0.3 mm. or more lateral, and at least 0.4 mm. posterior to it possesses the maximum medullary-producing capacity at the definitive primitive-streak stage. The results obtained in the present study suggest that this same area is concerned with the production of pigment.

DuShane (1934, 1935) has shown that the neural crest produces melanophores in *Amblystoma*, and Dorris (1936, 1938*a*, 1938*b*, 1939) demonstrated that neural-crest

cells of young chick embryos produce pigment when cultured *in vitro* and when transplanted to 3-day chick embryos. Eastlick (1939*b*) has corroborated this latter observation and also has demonstrated that limb buds of 60–70-hour Barred Plymouth Rock embryos develop noncolored plumage when cut free at the level of the posterior cardinal vein and grafted into the coelom of host embyros. Limb buds of $3\frac{1}{2}$–4-day embryos, similarly removed, invariably develop pigmented down. Apparently this is due to the migration of the neural-crest cells into the limb bud at the latter stages.

It is apparent that the pigment-forming area of the blastoderm, before the development of the neural crest, exceeds its prospective value. However, with the appearance of the crest, pigment formation seems to be taken over entirely by this derivative of the nervous system.

SUMMARY AND CONCLUSIONS

1. This study has revealed that various pieces of the blastoderm of Barred Plymouth Rock embryos in the definitive primitive-streak stage differ greatly in their capacity to produce melanophores when transplanted to the lateral body wall or into the intra-embryonic coelom of White Leghorn embryos.

2. Each transplant was found to possess a specific developmental potency which manifests itself in (*a*) the frequency of pigment production, (*b*) the extent of the pigmented patch, and (*c*) the intensity of pigmentation of the colored area.

3. It has been demonstrated that the region which contains the node possesses the maximum ability to produce pigment. Portions of the blastoderm more than 0.35 mm. anterior, or 0.25 mm. lateral, to the pit possess restricted potency, as shown by the fact that few positive cases were secured, and the pigmented areas were small. All pieces of the blastoderm 0.4 mm. or more posterior to the node give negative results.

4. The results suggest that the pigment-forming area coincides with the regions which have been shown by Hunt, Rawles, Rudnick, and others to differentiate medullary tissue in chorioallantoic grafts or in explants.

5. Few successful grafts were secured when pieces of the blastoderm, large or small, were placed into the intra-embryonic coelom.

6. In two cases the middle third of 8-hour blastoderms has been found to produce small pigmented areas.

While this paper was in press, a report entitled "The pigment-forming potency of early chick blastoderms" by M. E. Rawles appeared in the *Proceedings of the National Academy of Sciences*, **26**:86–94, 1940. The author studied the pigment-producing capacity of unincubated blastoderms and stages through eight somites. The results which she obtained with fragments of primitive streak stages seem to be similar to those found in the foregoing report. Her experiments show conclusively that the pigment-producing areas of early chick blastoderms coincide with the areas which develop nervous tissue in chorioallantoic grafts.

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PLATE I

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PLATE I

FIG. 4.—Piece 2 in Series A transplanted into intra-embryonic coelom. Note large mass loosely attached to mesenteries bearing colored plumage. Transplants of same region grafted to lateral body wall usually produced large heavily pigmented areas.

FIG. 5.—Piece *B* in Series B transplanted to body wall. Large pigmented area resulted. Node region consistently produced extensive heavily pigmented areas.

FIG. 6.—Piece *C* in Series B transplanted to body wall. Posterior half of node consistently pigmented many feathers of host.

FIG. 7.—Piece 2 in Series C transplanted to lateral body wall. Middle pieces of blastoderm consistently produced higher percentage of positive cases and more extensive colored areas than literal pieces of the same level.

FIG. 8.—Piece 6 of Series C transplanted to body wall. A few pigmented feathers resulted. Right lateral pieces seemed to have lower potency than corresponding left portions.

FIG. 9.—Piece 7 of Series C transplanted to body wall. Rather large pigmented area shown. The other positive case possessed a smaller colored area.

THE EFFECT OF EXCESS POTASSIUM ON THE CORTEX OF THE FERTILIZED EGG OF ARBACIA PUNCTULATA

(Two plates)

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IT seldom becomes possible to study a single reaction system of protoplasm inside the living cell. However, one of us (Moser, 1939*a* and 1939*b*) has recently succeeded in doing this for the cortical-layer granules of the unfertilized *Arbacia* egg. He was able to show that all sorts of stimulating agents (sperm, mechanical, radiational, electrical, and chemical) cause these surface granules to break down in a characteristic response leading to elevation of the fertilization membrane. Here, then, one possesses an extremely reactive protoplasmic system whose biochemical and physiological properties can to some extent be studied.

It is the purpose of this paper, the first of a series, to report upon another protoplasmic system available for experimental attack. This consists of the red-pigment granules of the living, fertilized *Arbacia* egg. The function of these granules is not known. One very interesting feature of their behavior has been remarked upon by several observers. This is the fact that the chromatophores, which lie freely distributed in the interior of the unfertilized egg, become localized in a differentiated cortex after having migrated peripherally to this region within 10 minutes (monaster stage) after fertilization (Harvey, 1910). This peculiar cytological behavior of the chromatophores is paralleled by their susceptibility to certain chemicals. It will be shown that certain reagents cause these granules to "explode" and to release the pigment from the living, intact egg. However, these reagents are not effective on the red granules in the unfertilized egg or, indeed, on those of the fertilized egg until about the time of the formation of the monaster.

It is therefore obvious that, even as the reaction of the surface granules reflects the properties of the cortex of the unfertilized egg, so the reaction of the red-pigment granules may be used to interpret the biochemical properties of the fertilized egg cortex. Fortunately, in spite of our ignorance of the physiology of the chromatophores, we do possess some information concerning the chemical conditions which cause these granules to become unstable and to disintegrate. Heilbrunn (1934) has shown that they release their pigment in the presence of free calcium or magnesium ions (surface precipitation reaction), calcium being perhaps one hundred times as effective as magnesium. When eggs are crushed in isotonic solutions of sodium, potassium, or calcium—binding agents, like citrate or oxalate—the red chromatophores remain intact. In other words, the breakdown of the chromatophores may be used as a criterion of the presence of free calcium or magnesium ions. We assume, therefore, that when a reagent like potassium (which, as noted above, has no effect on the isolated pigment granules) causes the chromatophores to break down in the living egg, it does so in an indirect manner, namely, by releasing calcium or magnesium from bound combinations. As previously

noted, the breakdown of the chromatophores takes place only after they have become definitely localized in the outer stiff cortex of the cell. This fact is additional evidence that it is the cortex which contains the masked calcium compound.

METHODS

Two methods have been employed to demonstrate the effects to be described. One is macroscopical; the other, microscopical. In general, the microscopical method, being much more difficult, was used but seldom. It serves as a check on the observations made by the macroscopical technique. In any case, certain precautions must be observed. Thus, it is necessary to avoid contamination of the egg suspension by the yellow perivisceral fluid. For this reason eggs should not be procured by permitting the urchins to shed, but the ovaries should be removed from the test. The ovarial tissue must be discarded immediately after the eggs have been collected. The egg suspension is then filtered through a double thickness of fine cheesecloth.

The experimental reagents consisted of three isotonic potassium salts. These were 0.53 M potassium chloride, 0.35 M potassium citrate, and 0.25 M and 0.30 M potassium oxalate. The isotonicity was tested by immersing the eggs in these solutions and observing that neither shrinking nor swelling occurred.

In both the macroscopical and the microscopical methods the proportion of reagent to egg suspension was alike, namely, 1:1. The macroscopical method was carried out as follows. Into the first of a dozen small test tubes, set in a rack, was placed a pipette-full of a moderately thick suspension of unfertilized eggs. To this was added an equal volume of one of the experimental solutions listed above, and the tube was inverted once to insure mixing. The color of the supernatant fluid could be determined almost immediately. The preparation of the second tube was carried out in the same way, except that the eggs had been fertilized. Such a tube could be prepared 1 or 2 minutes after fertilization. Five minutes later (i.e., about 7 minutes after insemination) a third tube was set up; then a fourth (12 minutes after fertilization); and so on. The last two or three tubes were prepared with eggs in the two-, three-, or many-celled stage, or even blastulae. The entire series was then photographed in color with the help of Mr. E. P. Little.

The microscopical ("drop") method consists in first placing a very small drop of egg suspension on a slide without adding a cover slip. A single egg was quickly selected for study with the high dry objective. To the edge of this drop was added an equal volume of the experimental solution. This diffused rapidly throughout the system, as was evident from the diffusion and convection currents set up around the egg that was being observed. The resultant changes of the red-pigment granules within the cell were recorded by photomicrographs and by a motion-picture apparatus kindly placed at our disposal by Dr. D. L. Hopkins, of Duke University, to whom we are greatly indebted for this service. Observations were made under light- and dark-field illumination.

OBSERVATIONS

If one carries out the macroscopical procedure outlined above, using 1 part of isotonic potassium salt to 1 part of egg suspension, one gets a series of colorless and colored superatant fluids, as illustrated in Plate I. At the bottom of each test tube may be seen the layer formed by the eggs that have settled. The first tube (starting on the left)

contains unfertilized eggs, and the supernatant fluid is colorless. The next three test tubes also show colorless supernatant fluids. These contain fertilized eggs: the first prepared 2 minutes; the second, 5 minutes; and the third, 8 minutes, after fertilization. All the other tubes (the fifth set up 11 minutes after fertilization) show "orange" supernatant fluids. It is important to note that the color appears *instantaneously;* it is apparent even as the eggs are settling after inverting the mixture.

If, now, one examines the colored supernatant fluid under a microscope, it is found to consist of a clear transparent solution *which never contains any cytolyzed eggs.* In other words, the color is due to the release of the echinochrome pigment from the red granules of the living egg. Examination of the fertilized eggs in the KCl–sea-water mixture within an hour or two reveals (at the height of the season) 95–100 per cent first and second cleavages. Citrate and oxalate usually inhibit the cleavage of eggs left for so long a time in the tubes, although there can be no doubt that there is no toxic effect within the first 15–30 minutes. This must be so, since all the eggs reach the diaster stage. As a matter of fact, this toxic effect is no doubt in some measure due to CO_2 accumulation, because eggs immersed in less dense suspensions show a much higher percentage of successful cleavages.

The foregoing effects are substantiated by the "drop" method. When a drop of 0.53 M KCl is added to a drop of unfertilized eggs under the microscope, nothing happens. Nor is there a reaction if the KCl solution is added to eggs 5–8 minutes after being fertilized. But, if a drop of the KCl solution is added to eggs that are in the monaster stage (about 12 minutes after fertilization), a striking phenomenon occurs. At this time the red-pigment granules have reached the cortex (Harvey, 1910; Brown, 1934). As one focuses on this cortical layer while the KCl solution is diffusing to the egg, a sudden "explosion" of these granules occurs, and they disappear from view. This is shown in Plate II, which represents a series of motion-picture frames of the exploding red-pigment granules of a single, centrifuged, fertilized egg. (The disappearance of individual red granules can easily be made out by concentrating one's attention on the groupings in the hyaline area. Even a casual glance at the hyaline areas of photomicrographs 1 and 33 shows the sequence of disappearances.) It is important to note that the pigment always escapes to the exterior from the living egg and is never trapped within it, as under conditions of so-called "pale" cytolysis. An important feature of this reaction is the fact that only a few chromatophores break down when the eggs are first immersed in the experimental solution. As a matter of fact, examination of eggs in a KCl–sea-water mixture at the fourth or fifth cleavage reveals the fact that there are a few red granules still present in the blastomeres. They seem to be especially stable when imbedded in the cleavage furrow region.

It may be noted that none of the salts is effective on the chromatophores of the unfertilized egg. This is true even if the cells are permitted to remain an hour or more in the experimental solutions. On the other hand, the ovarial tissue itself may give a slightly colored supernatant fluid. For this reason contamination must be assiduously avoided.

Furthermore, it appears that up to about 10 minutes after fertilization pigment is not released by these reagents. This phase of nonsusceptibility, however, is not repeated during the first 10 minutes of the second, third, or successive cleavages. Once the new fertilized egg cortex is established, the pigment can be released by these agents

at any time during early development. In oxalate the color can be recognized even through the great crop of white CaC_2O_4 crystals that forms as soon as the potassium oxalate is added.

DISCUSSION

As stated in the introduction, it is the purpose of this paper to describe a reaction which is capable of revealing the presence of free calcium ions in the cortex of the living, fertilized *Arbacia* egg. That this reaction is restricted to the cortical region of the cell is based upon the following evidences. Isotonic KCl has absolutely no effect upon the pigment granules of the unfertilized egg. Furthermore, this solution has no effect upon the pigment granules of fertilized eggs before the formation of the monaster (about 10 minutes after fertilization). In both these cases the pigment granules are distributed uniformly throughout the cell interior. However, once the fertilized egg has reached the monaster stage, isotonic KCl in sea water becomes effective in breaking down the chromatophores. The important thing to note here is the fact that at this time the pigment granules are no longer freely dispersed in the interior of the cell but have now become almost entirely localized in a new cortex. Bearing in mind the fact that potassium has no effect on the isolated pigment granules, we are led to the conclusion that the KCl solution reacts with the "ground substance" in which the pigment granules are imbedded, rather than directly on the pigment granules themselves.

Concerning the evidence for calcium release by the potassium ion, we have already presented the qualitative aspects as ascertained from the surface precipitation reaction of Heilbrunn. Actually, quantitative data exist (Mazia, 1937, pp. 299–300) which indicate that media low in calcium (potassium chloride and potassium citrate) are effective in causing an increase in free calcium in the *Arbacia* egg—presumably by releasing bound calcium from the cortex. The conclusion that the fertilized egg cortex consists, to some extent, of a masked calcium compound is inescapable.

The preceding paragraphs have been devoted to enumerating the evidences for ion release in a definite protoplasmic region of the living cell. This phenomenon offers, however, additional points of interest. Some of these we should like to mention briefly. In the first place, the phenomenon of "exploding" cells or cell granules is of considerable physiological importance. There is, of course, an extensive literature on exploding platelets in the field of mammalian blood-clotting. More important for our discussion, perhaps, are the observations of Donnellon (1938) on the nature of the clotting of the perivisceral fluid of the sea urchin. He found that the formation of the "cell clot" is accompanied by an explosion of the red granules of the "red amoebocytes" and of the "colorless" granules of the "white" ones. Moreover, there is a biochemical parallelism in that isotonic KCl is especially effective in causing granule breakdown.

Perhaps the most interesting feature of this work lies in the fact that it represents a clear case of a difference in the physiological behavior of the cell surface at different times after fertilization. For, whereas much attention has been devoted to demonstrating differences in the physiological properties of the cell before and after fertilization, comparatively little stress has been laid upon the fact that the properties of the cell surface are progressively changing following insemination. There are several indications in the literature that this is so. Thus, Kopac (1938), studying the phenomenon of coalescence of *Arbacia* eggs with oil drops, remarks: "The coalescencies of fertilized eggs

if kept in sea water decrease steadily and become zero after 15 or more minutes." With respect to water permeability, Lucké and McCutcheon (1932, p. 123) state:

> Returning to fertilized *Arbacia* eggs it was found that increase in permeability is not instantaneous and is in greater part completed during the next 10 minutes. This was demonstrated by transferring eggs to hypotonic sea water at different intervals after insemination. During the first few minutes after sperm has been added, eggs in hyperfonic solutions shrink slowly and remain round like fertilized eggs. After 5 to 6 minutes from the time of insemination, noticeable increase in rate of shrinking occurs and this becomes more rapid at the same time that the tendency to crenation becomes more pronounced. Increased water permeability appears to remain as a permanent quality of the developing egg and may readily be demonstrated in the 2-cell and 4-cell stages in intervals between cleavage.

It is therefore clear that, following insemination, the cell surface is changing and progressively approaching some sort of equilibrium which is attained about the time of monaster formation.

With respect to the permeability characteristics of the cell surface at this time, an interesting problem is encountered in attempting to explain the instantaneous egress of the pigment from the living cell. Dialysis experiments with collodion membranes indicate that the pigment may be of high molecular weight, since it does not dialyze over a considerable period of time.[1] (Lederer and Glaser, 1938, assign the following empirical formula to the echinochrome extracted from the ovaries of *Arbacia aequituberculata:* $C_{12}H_{10}O_7$.) The question arises as to the mechanism whereby this large molecule passes out of the living cell. Eggs immersed in the pigment do not take it up—i.e., the cell does not seem to be freely permeable to it. Furthermore, why does the pigment diffuse out of, rather than into, the interior of the egg? To these and other interesting questions raised during the course of this investigation we are at present unable to give an answer. We may remark, however, that the properties of the cell surface appear to find highly suggestive analogies in the systems of protein and fatty-acid films described by Langmuir, Schaeffer, and Wrinch (1937).

SUMMARY

The red-pigment granules of the *Arbacia* egg have been used as indicators of ionic changes in the cell cortex. It is known that these granules disintegrate in the presence of free calcium ions but remain intact in isotonic potassium solutions or when treated with oxalate or citrate. When unfertilized eggs, or fertilized eggs that have not reached the monaster stage, are immersed in 1:1 proportions of isotonic potassium chloride, potassium oxalate, or potassium citrate to sea water, nothing happens to the chromatophores. This, in accordance with the foregoing observations, was to be expected. However, when fertilized eggs, in which the red granules are localized at the cell cortex (monaster stage), are treated with these reagents, many of the red granules disintegrate and release their pigment to the exterior. The cells remain alive and intact; there is no cytolysis. We conclude (1) that after the monaster stage the cortex of the fertilized egg

[1] The appearance of a faint green color in the dialysate is an indication that the orange pigment released by the action of potassium may consist of a mixture of pigments. In this respect we quote from Glaser and Lederer (1939), who find that "dans *Arbacia*, l'échinochrome est accompagné d'un pigment plus foncé l'*iso-échinochrome* (fines aiguilles brun rouge; F. 247°; mieux adsorbé; soluble dans l'éther; bandes d'absorption à 548 et 510 mμ dans $CHCl_3$), et d'un deuxième pigment (insoluble dans l'éther, soluble dans l'alcool butylique; bandes d'absorption, 515 et 480 mμ dans l'acétate d'éthyle)."

contains as a component a masked calcium compound and (2) that potassium can release this bound calcium, which then causes granule breakdown. In addition, brief references have been made to various aspects of this phenomenon which may be of interest to the cellular physiologist. Especial stress has been laid on the possibility of analyzing the biochemical and physiological properties of the cell cortex by the procedures outlined.

We are very happy to thank Dr. L. V. Heilbrunn for his unfailing interest, encouragement, and ready counsel.

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PLATE I

Photographs of test tubes containing unfertilized and fertilized eggs at various stages of early development after treatment with an isotonic KCl solution (see text). The layer of cells can be seen at the bottom of each tube. The color of the supernatant fluid changes upon standing. Furthermore, the pigment assumes different colors in the presence of different ions. For a study of the color chemistry of such systems the reader is referred to a paper by Vlès, Gex, and Riess (1928). A detailed discussion of the conditions under which the living eggs release their pigment to the supernatant fluid is presented under "Observations."

PLATE II

Selected stages from photomicrographs (unretouched) of the breakdown of the red-pigment granules of a centrifuged, fertilized *Arbacia* egg when treated with an isotonic KCl solution (see text). The focus is upon the upper surface of the hyaline area, wherein the red chromatophores are imbedded. The large black "dots" are the red-pigment granules, and their successive disintegration can easily be traced. It is interesting to note that these "granules" are not uniform in shape or in size. The prints were made, as numbered, from motion-picture negatives taken at standard speed, 16 frames per second.

PLATE I

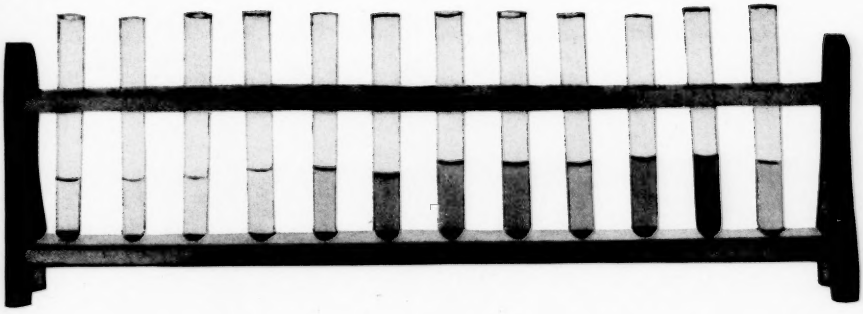

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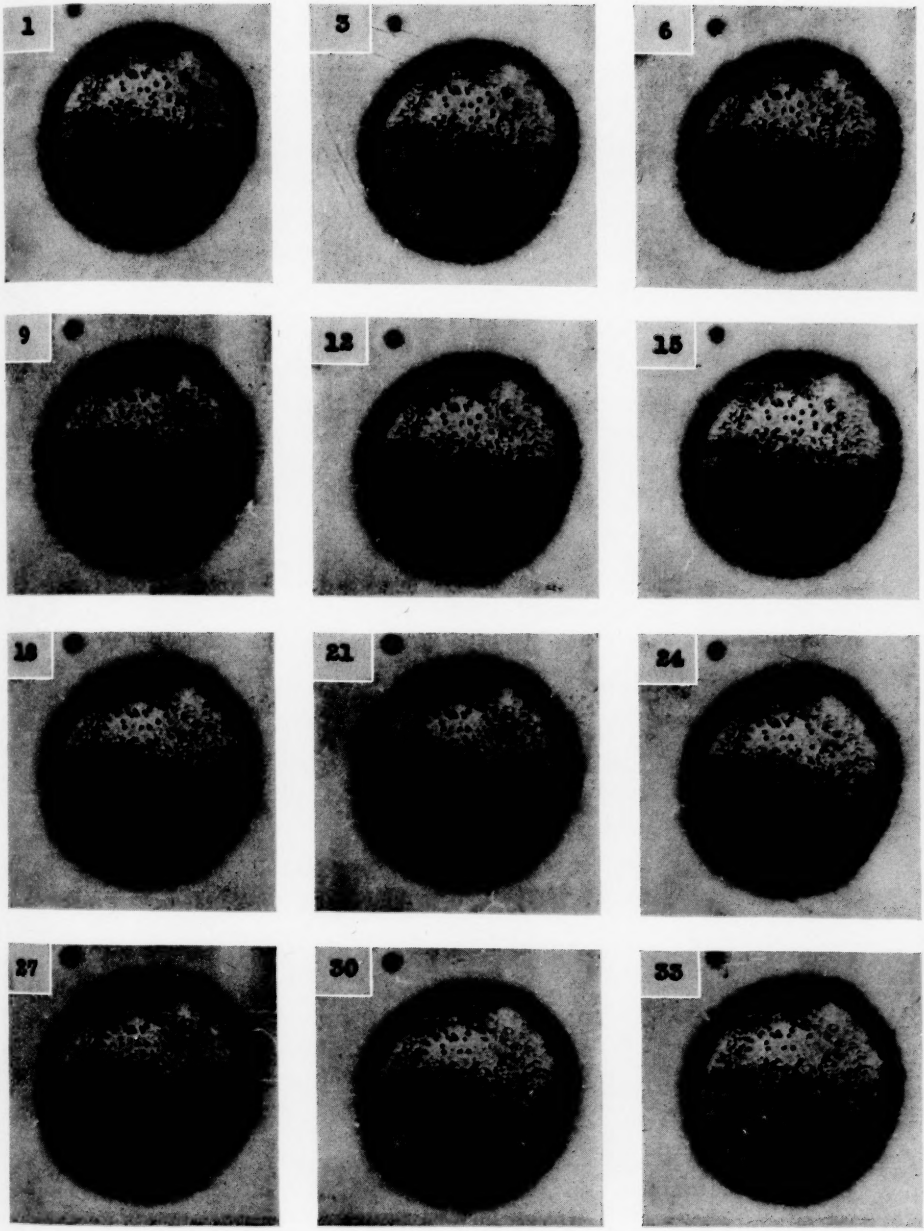
PLATE II



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THE EFFECT OF THERMAL INCREMENTS ON AND THE SUBSEQUENT ADJUSTMENT OF THE PROTOPLASMIC VISCOSITY OF AMOEBA PROTEUS

(Two figures)

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TEMPERATURE is one of the most thoroughly investigated variables affecting biological systems. While most investigations have been concerned with thermal increments which are relatively slowly applied, it has been impressed infrequently upon the investigator that thermal increments which are suddenly applied are to be interpreted, on the basis of experimental results, differently from those which are less rapidly applied. Thermal changes which are applied suddenly within the biokinetic range, and possibly those increments of a potentially lethal nature (e.g., "superoptimal") which are applied for very short periods of time, are to be considered as stimulative in character.

In the case of single cells Dutrochet and Becquerel (1837), working on the alga, *Chara fragilis*, were the first investigators to record the effect of suddenly applied thermal changes. Since this work, most of the investigations have been concerned with botanical forms in which suddenly applied thermal increments may cause a temporary cessation, retardation, or acceleration in the speed of protoplasmic streaming. The monograph of Ewart (1903) should be consulted for a review of this phase of the literature. In view of the present state of our knowledge and of Heilbronn's experiments (1914) it is inopportune for one to interpret cyclosis in terms of viscosity data and to attempt an appraisal of the literature on sudden thermal changes on cyclosis in the light of the present studies.

Little direct work has been undertaken with the view of suddenly applying thermal increments on animal cells with the exception, perhaps, of inducing artificial parthenogenesis in certain invertebrate eggs, though here, apparently, other factors are to be considered. A review of the literature in these fields may be obtained from chapter xi of Bělehrádek's book (1935). Recently Hopkins (1937) has studied the effect of adaptation to temperature in the locomotion-rate of the marine amoeba, *Flabellula mira*.

This investigation was originally planned to show the effect of sudden application of, and recovery from, heat when applied, within the biokinetic range, on the highly labile plasmagel of *Amoeba proteus*. The preliminary experiments were performed during the Thanksgiving recess (1936) in Dr. D. L. Hopkins'[2] laboratory at Duke University. It was Dr. Hopkins' intention to correlate the rate of locomotion with viscosity data; but, as monopodal amoebae invariably underwent polypodality at some time during experimentation, measurements of the locomotion-rate obviously were futile.

[1] This investigation was aided by a grant from the Faculty Research Committee, University of Pennsylvania.

I wish to thank Professor C. E. McClung for his many kindnesses during the course of this work. I also wish to express my gratitude to him and to Dr. E. R. Helwig for their kind co-operation in arranging for me to use their much-needed temperature room.

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[2] I wish to thank Dr. D. L. Hopkins for the facilities placed at my disposal during my sojourn in his laboratory.

MATERIAL AND METHODS

The material employed in this investigation was *A. proteus;* the specific cytoplasmic layer studied was the plasmagel. Amoeba cultures were prepared from stocks originally purchased from the Carolina Biological Supply Company and from Professor A. A. Schaeffer. Amoebae were cultured in 4-inch finger bowls to which quartz distilled water was added until a depth of about 15 mm. was obtained. To each culture was added two or three sterile wheat grains plus a few drops of culture fluid containing *Chilomonas*. The cultures were then placed in a constant-temperature cabinet. After 3 or 4 weeks the wheat grains were removed, and the cultures were tested periodically for "normal" viscosity. The temperature cabinet was maintained at a fairly constant temperature at least 5 days prior to experimentation. The temperature did not fluctuate more than 3° C. during this period. Likewise the pH of the culture medium was maintained between 6.6 and 6.8.

The viscosity of the protoplasm was determined by the centrifuge method. An Emerson hand centrifuge, which developed a centrifugal force of 2,474 times gravity when the handle was turned at the rate of one revolution per 2 seconds, was employed in these experiments. The end-point used in comparing the viscosity of the experimentally treated with the control or "normal" amoebae was the time in seconds necessary for a constantly applied centrifugal force to move the intracellular crystals into one-half of the more or less spherical cell. The time in seconds necessary to achieve this end-point is known as the "centrifuging value."

The experiments were performed in the following manner. Amoebae were reared at a fairly constant temperature $\pm 1°.5$ C. and pH 6.6–6.8 for a 5-day period prior to experimentation. The initial temperature to be employed was determined from the nature of the experiment in question. All temperatures studied were within the biokinetic range for culturing amoebae. When any amoebae culture was ready for experimental use, the culture bowl, containing amoebae, was placed in a glass receptacle, the inner diameter of which was 7.5 inches; concentric with this was a larger glass vessel, the diameter of which was 9 inches. The space between the two concentric glass vessels was packed with thoroughly dried sawdust and sealed by a plastic cement. On the bottom surface there was an area of such dimensions as to permit transmitted light to illuminate the culture when under a large-field binocular microscope. This jacket served to prevent currents of air from exerting any sudden change of temperature on the undersurface of the culture bowl to which amoebae were generally attached. Thus, all surfaces of amoebae were exposed to approximately the same temperature, as indicated.

Upon removal from the constant-temperature cabinet the culture bowl containing amoebae was placed in water of the same temperature as that of the culture medium. When 80 per cent, or slightly more, of the amoebae showed a centrifuging value between 140 and 160, the amoebae were declared "normal" and were ready for experimental purposes. Amoebae were then removed to the thermostatically controlled temperature room. The procedure then followed either one of two methods according to the nature of the experiment in question.

a) Slowly applied thermal increments, e.g., 11°.6–26° C./ca. 200 minutes (Fig. 1).—The culture bowl, immersed in the larger double-jacket glass vessel containing water at the same temperature as the cabinet, was placed in a constant-temperature room with thermometers immersed respectively in the culture and jacket fluids. The experiment was

timed from the moment the culture was brought into the higher-temperature room. Centrifuge tests were made consecutively, and the rate of increase of the temperature in jacket and culture fluid was recorded immediately prior to each centrifuging. The temperature differential between the two fluids was never greater than 1° C. Centrifuge tests were made in an anteroom, the temperature of which was initially comparable to that of the cabinet temperature and was permitted to increase synchronously with the thermal increment to which the amoeba culture was experimentally subjected. While the method did not permit perfect synchronization between anteroom and culture temperatures, it was possible to keep them within 3° C. (maximum) of each other. This differential between culture and centrifuging temperatures does not serve to stimulate the plasmagel.

b) Suddenly applied thermal increments, e.g., 10° C. or more rise in temperature within 1–10 minutes (Fig. 2).—The same procedure was followed as described under (*a*), only the temperature of the culture medium was increased to within 2° or 3° of room temperature by the addition of preheated culture medium which was never warmer than 30° C., and thus below the lethal temperature for this species of *Amoeba* (see Thornton, 1932, 1935). The time was recorded from the initial application of the warmed, culture medium to the culture bowl. For slower application of temperature (e.g., curve *B*, Fig. 2) preheated culture fluid was added at such a rate that the desired temperature was attained progressively over a 10-minute period. In all cases the fluid in the various culture bowls was gently stirred as the warmed culture fluid was slowly added.

In developing a curve, eight to twelve amoebae were centrifuged at a value slightly higher than that anticipated on the bases of preliminary experiments. As fast as one could work, this point was investigated until the desired end-point was obtained. After every positive (end-point appearing in over 80 per cent and less than 100 per cent of the centrifuged amoebae) centrifuging value was obtained for those portions of the curves (Figs. 1 and 2) which show rapid change of value, a further centrifuge test was employed in order to determine that the positive value represented was succeeded, in turn, by one of negative character. Succeeding centrifuge values were determined in a comparable manner.

By employing the technique already described, the viscosity curves (Fig. 1) were investigated at six different times, while the 2-hour studies of the curves which represent over a 10° C. change within 1–10 minutes (Fig. 2, curves *A* and *B*) were performed seventeen times, and those curves showing recovery (Fig. 2, curve *A'*) were studied five times. The recovery curves were always investigated immediately after the temperature had been increased to within 1°–2° C. of the new thermal environment. All experiments represent different amoebae cultures, while curves *A*, *B*, and *A'* represent data as plotted from one stock culture.

RESULTS

a) Slowly applied thermal increments, e.g., 11°.6–26°. C./ca. 200 minutes (Fig. 1).—When one subjects *A. proteus* to a thermal increment as represented by the broken-line curve the co-ordinates of which represent the time-rate of thermal increase, it is found that there is a progressive decrease (solid-line curve) in the centrifuging value for each arbitrary progressive increase in temperature. The solid-line curve, representing data as obtained for one amoebae culture, shows the centrifuging value (viscosity) plotted as a function of the time of exposure in minutes to the thermal increment as expressed by

the broken-line curve. In the experiments thus recorded, starting with a culture temperature of 11°.6 C. and a centrifuging value of 140 seconds, the temperature increases in 58 minutes to 18°.6 C., while the centrifuging value decreases simultaneously to 90 seconds, i.e., a 36 per cent decrease; after 156 minutes the temperature increases to 24°.3 C. and the viscosity decreases simultaneously to a centrifuging value of 40 seconds. Finally the culture temperature increases and levels at 26° C.; the centrifuging value levels at 40 seconds. When the temperature is maintained at this value, the viscosity does not change within 8 hours after the beginning of the experiment, nor when a determination is made the following day (about 24 hours after the start of the experiment).

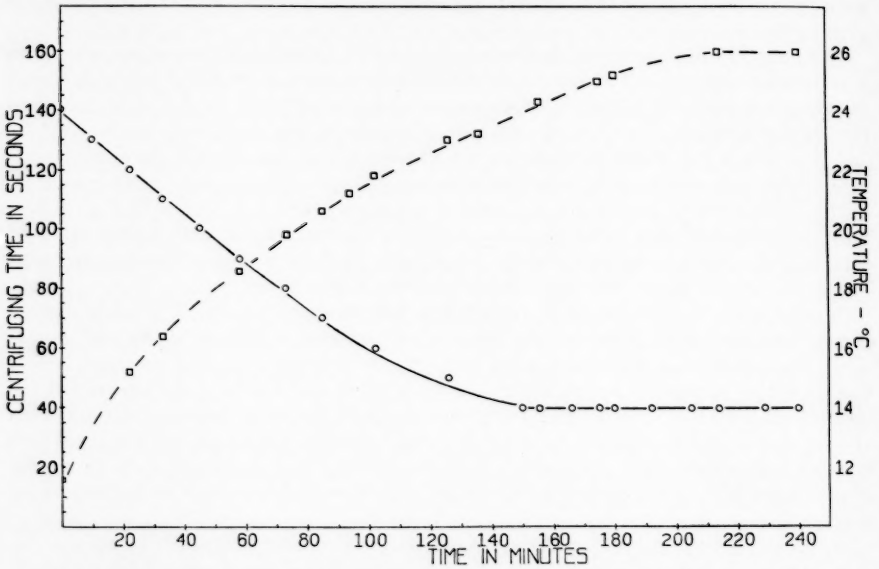

FIG. 1.—The solid-line curve (○) represents the centrifuging time in seconds (viscosity) of the plasmagel of *A. proteus* versus time in minutes during which the viscosity is subjected simultaneously to the thermal increment as represented by the broken-line (□) curve. The latter curve shows the time-rate of thermal increase to which the amoeba culture is subjected. pH, 6.6—6.8.

b) Suddenly applied thermal increments, e.g., 10° C. or more rise in temperature within 1–10 minutes (*Fig. 2*).—The data for two experiments are represented by the curves in which centrifuging values are plotted as functions of the time in minutes during which the viscosity of amoebae was investigated when the amoeba culture was subjected to thermal increments of 10° or more above room temperature within an interval of 1–10 minutes.

Curve *A* shows the results of one experiment in which the culture temperature was raised from 16° C. to a final value of 27° C. within 10 minutes. After a lapse of 11 minutes from incipient temperature increase, the centrifuging value decreases 37 per cent, while 24.5 minutes later there is a 75 per cent decrease from the "normal," or control, viscosity value. Further lapse of time finds no apparent change in the centrifuging value

until after 38 minutes, when the viscosity progressively increases; and at 109 minutes the centrifuging value has gained 275 per cent over its previous minimum value.

Curve *B* (Fig. 2) represents another amoeba culture which was brought from a temperature of 12° C. to 26° C. within 1 minute. Thus the decrement in the centrifuging value is steeper (cf. curves *A* and *B*), so that after 3 minutes from the incipient thermal increase the viscosity has decreased 62 per cent, while after 9 minutes a decrease of 81 per cent is attained. Similarly, the viscosity levels at a transient minimum value which

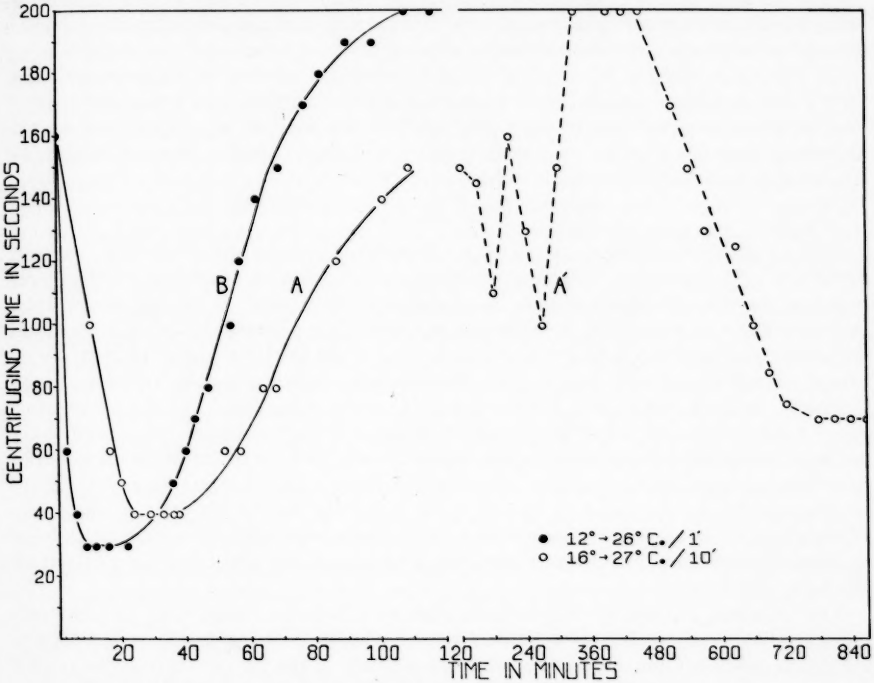

FIG. 2.—Centrifuging time in seconds (viscosity) of the plasmagel of *A. proteus* versus time in minutes from the initial exposure to the thermal increment thus indicated. Curve *A'* is a continuation of curve *A*. pH, 6.6–6.8.

is maintained for approximately the next 13 minutes, after which the centrifuging value progressively increases with time. After 116 minutes it has increased 566 per cent from the previous minimum value.

Curve *A'*, a continuation of curve *A* (Fig. 2), shows the fluctuations in the centrifuging value when a study is undertaken of the period of recovery from the application of a relatively steep thermal increment. These data were not obtained on a minute-to-minute basis, as in the 120-minute curves (represented by curves *A* and *B*, Fig. 2), but during 15–30-minute intervals after the termination of the initial 120-minute interval. It was recognized, even on the basis of preliminary experiments, that after the first minimum and

maximum all subsequent minima and maxima, representing the fluctuating centrifuging values, bear no constant relation to each other when different experiments, i.e., different amoeba cultures, are compared with respect to the time (abscissas) at which these values are respectively attained. The first minimum was always quantitatively reproducible within the limits of experimental error; the first maximum was predictable as regards the time variable (abscissas) but not with respect to the viscosity value (ordinates). Hereafter the fluctuations were predictable as such, but never from a quantitative viewpoint with regard to time or centrifuging value. Within $9\frac{1}{2}$–14 hours the plasmagel assumed a constant centrifuging value, which was maintained the following day (approximately 10–12 hours after close of the experiment.)

In three experiments in which a thermal increment of shorter temperature range (5° C. increase above cabinet temperature) was applied within the shortest time possible (1 minute) under conditions of the technique here described, it was found that such an increment does not produce results comparable to those of greater thermal magnitude, i.e., an increase of 10° C. or more.

DISCUSSION

When the plasmagel of *A. proteus* is subjected to a relatively slow thermal increase, e.g., from 11°.6 C. to 26° C. within approximately 200 minutes, there is a progressive decrease in the viscosity which more or less parallels synchronously the progressive increase in temperature to which the amoebae are exposed. It is common knowledge that most inanimate gels undergo a progressive liquefaction when subjected to a temperature increase. Similarly, in the majority of experiments on protoplasmic systems, Fauré-Fremiet (1913); Weber (1916), Pantin (1924), Costello (1934), and, for the temperature range as herein studied, Thornton (1935), working on the same species, find a progressive decrease in the viscosity as the temperature increases. In view of our limited knowledge as to the factor or factors involved in, and the criticism of Heilbronn (1914) with regard to, the relation of the speed of chloroplastid streaming to the viscosity of the protoplasmic system in question, it would be superfluous to attempt any analogy. A review of this field may be obtained from Ewart (1903) for the earlier, and Bělehrádek (1935) for the later, literature.

The apparent incongruity between the time at which the temperature and the centrifuging-value curves (Fig. 1) approach asymptotically their respective maximum and minimum ordinates may be ascribed at least partially to the lag between the successive temperature changes, as induced in the culture fluid, and their transmission to the protoplasm of amoebae. Perhaps the greater factor is that, as the temperature and viscosity respectively approach their constant or leveling values, they do so by increasingly smaller and smaller increments. Thus, considering the experimental error inherent in the centrifuge method, a difference as produced in the viscosity by 1° of temperature is impossible of direct measurement.

The primary reason for which these experiments were pursued was to study the behavior of the plasmagel to suddenly applied thermal increments when acting within the biokinetic range. Thermal increments that are more suddenly applied (Fig. 2) produce steeper decrements in viscosity, and the respective minimum centrifuging value attained is lower the more suddenly the thermal increment is applied, and, within the limits of the temperature range herein explored, is independent of the value to which the temperature is experimentally elevated. Although there is no literature apparently on this subject,

except the study of Hopkins (1937) on the adaptation of *F. mira* to temperature and that based on the chloroplastid streaming method, nevertheless there is an analogous behavior when one compares the action of various stimulating agents on the plasmagel of *A. proteus*. That this layer is a highly thixotropic gel may be concluded from the experiments of Angerer (1936); and that various stimulating agents will cause a transitory liquefaction of this layer is shown by the action of such stimulating agents as ultra-violet radiations (Heilbrunn and Daugherty, 1933), mechanical agitation, electric current (Angerer, 1936, 1937), and now for relatively steep thermal increments when applied within the biokinetic range. Heilbrunn (1937), on the basis of his and his students' work, has developed a colloid chemical theory of stimulation which anticipates such a reaction for the plasmagel.

Hopkins (1937) has shown that in the adaptation of the locomotion-rate in *F. mira* to temperature there is a progressive adjustment when the thermal increments are protracted while a long period of fluctuation ensues when thermal increments are more suddenly applied. If we assume that the physical state of the protoplasmic colloid is the sole factor in determining the rate of locomotion in the amoeboid cell (Pantin, 1923; Mast, 1926), Hopkins' results are explicable on the basis of the viscosity data as presented here.

The nature of the physical-chemical change or changes manifesting themselves during the series of viscosity fluctuations prior to the assumption of a constant value characteristic of the temperature in question is problematical at this time. According to the colloid chemical theory of stimulation as developed to date, calcium is the principal substance to be identified with the reaction induced by certain stimulating agents. Recently Heilbrunn and Daugherty (1938) have found that fat is released when *A. proteus* is subjected to ultra-violet radiations. On subjecting *A. proteus* to mechanical impacts, and again during the course of these experiments, there appeared an increase in the number of highly refractive "globules" within the cytoplasm. During the course of this work the "globules" were found to stain with Sudan III, and thus may be identified as fat. However, since all cells observed have contained some "globules" prior to experimental treatment, it is suggestive to treat of this material in at least a semiquantitative manner. These experiments are planned for the future. On the basis of the evidence available at this time, it would indicate that during the sudden breakdown of the plasmagel, calcium and possibly fat, among other substances, are temporarily liberated when various stimulating agents are employed.

In considering Thornton's (1932, 1935) technique for a study of the temperature-viscosity curve for *A. proteus*, it was found that tap water (19° C.), when placed in the same type of centrifuge tube which Thornton employed in subjecting amoebae to the higher temperatures of the temperature bath, attained the temperature of the water bath in less than 1 minute for temperatures up to 35° C. Thus, it would seem that that part of the temperature-viscosity curve which embraces the higher temperature range of his curve should be treated with reservation.

Likewise, values for temperature characteristics (μ) and temperature coefficients (Q_{10}) based on a technique which induces fluctuations in the viscosity values as represented (Fig. 2) can have, obviously, little meaning. This same viewpoint is emphasized by Hopkins (1937) in considering the adaptation in locomotion to temperature by *F. mira*.

SUMMARY

1. The viscosity of the plasmagel of *A. proteus*, when subjected to various thermal increments, was determined by the centrifuge method.

2. Slowly applied thermal increments (e.g., 11°.6–26° C./*ca.* 200 minutes) produce simultaneously a correspondingly slow decrease in viscosity which levels at a value characteristic of the final temperature to which the amoeba culture is elevated.

3. On subjecting amoeba cultures to relatively steep thermal increments as applied here, there is produced a sudden decrement in viscosity which progressively decreases to a minimum value. The steeper the applied thermal increment employed, within the temperature range studied, the sharper the initial decrement and the lower the viscosity value attained.

4. When studying the recovery of the viscosity to an equilibrium value characteristic of the temperature in question, it is found that the viscosity passes through a succession of minima and maxima values. After the first minimum and maximum values are attained, there is quantitatively no predictable course through which the viscosity will pass, though 9½–14 hours are required before the viscosity of the plasmagel comes to a steady state.

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EXPERIMENTS WITH COLPIDIUM CAMPYLUM IN HIGH-FREQUENCY ELECTRIC AND MAGNETIC FIELDS

(8 figures)

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ONE has only to review the rapidly growing literature on the subject of radiotherapy to be impressed by the effectiveness of this form of treatment for a variety of human ills. However, from both laboratory and clinic have come several controversial questions relative to the nature and specificity of the action on tissues and organisms at different frequencies. As enumerated by Mortimer and Osborne (1935), the most important questions pertain to (1) degree and uniformity of penetration, (2) special selective thermal action, (3) specific biologic action, and (4) specific bactericidal action.

Of immediate interest to us is the problem of biological or bactericidal specificity upon which there is no unanimity of opinion at present. Haase and Schliephake (1931), Pflomm (1931), Liebesny (1932), Schliephake (1932), Stieböch (1933), Dausset and Dognon (1934), Jellinek (1936), and many others maintain that there are special effects of high-frequency fields which cannot be explained in terms of heat action alone. The other side of the picture is represented by an even longer list of competent observers who are unable to confirm heatless bactericidal action or any other specific electrical effect at high frequencies. With reference to the action of a 1–10 megacycles (Mc) electric field, Kovacs (1936) sums up this viewpoint: "Its primary physical effect is that of heat generation and this explains adequately its action" (p. 433).

We have approached this problem by preventing appreciable temperature variations during the exposure of individual protozoan organisms in minute quantities of media and by using the mean daily division-rate, as determined by the isolation culture method, as the criterion of general physiological condition after treatment. The data from these experiments comprise the substance of this report.

The work was done in the department of anatomy, Cornell University Medical College, New York City. We are greatly indebted to Professor J. B. Russell, of the electrical engineering department, Columbia University, for the use of two General Radio wavemeters, and to Mr. R. H. Hall, also of Columbia University, for the stock cultures of *Colpidium campylum*. It is also a pleasure to acknowledge the helpful suggestions made by Mr. Dale Pollack, of the R.C.A. Manufacturing Company; Dr. Myron A. Coler, of the Paragon Paint and Varnish Corporation; and Mr. W. W. van Roosbroeck, of Bell Telephone Laboratories.

PLAN OF STUDY, MATERIAL, METHODS

Colpidium campylum was selected for this work because it is an easily cultivated free-living type, small enough to become adjusted readily to temperature variations in the medium. It divides rapidly in drop cultures, and its structural and cyclical features are known fairly well (Sonneborn, 1932; Kidder and Diller, 1934).

Considerable attention was given to the maintenance of uniform environmental conditions throughout all phases of the work—salt concentration in the medium, temperature (25° C.), pH (5.1–5.3), and amount of inoculum transferred from culture to culture. The medium consisted of a rice-salt combination, R. H. Hall's modification (personal communication) of the formula given by Rohdenburg and Nagy (1937): KH_2PO_4, 0.5 gm.; KNO_3, 0.4 gm.; NaCl, 0.1 gm.; $MgSO_4$, 0.1 gm.; rice, 5.0 gm.; water, 1,000 cc. The mixture was boiled for 10 minutes, filtered through cotton, and allowed to stand in an open vessel for 24 hours before use. In order to standardize the procedure, a stock strain of *Colpidium* was established in isolation culture (five lines) and carried for 90 days before the first experimental lines were taken from it.

An important part of the procedure was the method of preparing individual colpidia for exposure in the high-frequency fields. One organism within a minute quantity of medium was centered in a thin-walled capillary, 4 cm. in length, with the aid of a microinjector. The ends were then sealed by flaming. Individual organisms incapsulated in this fashion were exposed for a standard time of 40 minutes. In all of the electric-field exposures, the capillaries were arranged in the center of the field, with long axes perpendicular to the field lines. For those runs in which thermal changes were to be avoided as far as possible, the capillaries were placed in the middle of a glass water jacket (Fig. 2), and the assembly lowered into the field. The control organisms were sealed under the same conditions but were placed in a 25° C. incubator for the 40-minute interval.

At each frequency setting, a number of incapsulated colpidia, all descendants of the single individual isolated 12 hours before, were exposed with water- and air-cooling. Twelve or more capillaries were prepared in the prescribed manner and divided equally into three groups. One group of at least 3 capillaries served as controls. The second group was treated for 40 minutes while in the water jacket. The jacket was then removed from the field, the frequency checked, and the third group exposed for a corresponding period without the water bath. The latter will be described as air-cooled exposures.

The capillaries were rinsed in alcohol, dried, and broken into fresh drops of medium for a 12-hour incubation period. Fission sometimes occurred within the capillary. In this event only one of the progeny was cultured. At the end of a 12-hour period there were usually between 16 and 32 organisms per drop. Five of these were isolated and continued in isolation cultures for at least 13 days. Thus, each experimental organism was propagated as a "series" of five lines. Although the number of divisions per day per series was computed on a 24-hour basis, we were obliged to isolate and to transfer this rapidly reproducing species at 12-hour intervals for the sake of accuracy in counting. At the forty-eighth hour all but one or two series from each of the three original groups were discontinued in order to make way for new series set up on successive days. Those continued were selected by serial numbers only. A total of 129 colpidia were treated in the high-frequency field during this investigation. Only 5 losses occurred, 3 of which could be attributed definitely to extraneous causes.

In another group of experiments, we were able to test the reactivity of incapsulated colpidia when subjected to either a homogeneous magnetic field or an inhomogeneous electric field at frequencies corresponding to those selected for the experiments already described. The possibility of an effect from a highly inhomogeneous field was suggested by the work of Kimball (1938), who reported a noticeable inhibition in the formation of yeast buds in a heterogeneous magnetic field, an effect that did not occur in a homo-

geneous field three thousand times more intense. Further, in a majority of practical applications, the radiotherapy field is not homogeneous. In our experiments only the electric field was made inhomogeneous.

We did not attempt to make exposures at frequencies higher than 60 Mc as this was the practical limit of the oscillator.

APPARATUS

Figure 1 is a schematic diagram of the apparatus designed to produce as pure a wave form as possible. The oscillator was a push-pull type in which all even-order harmonics

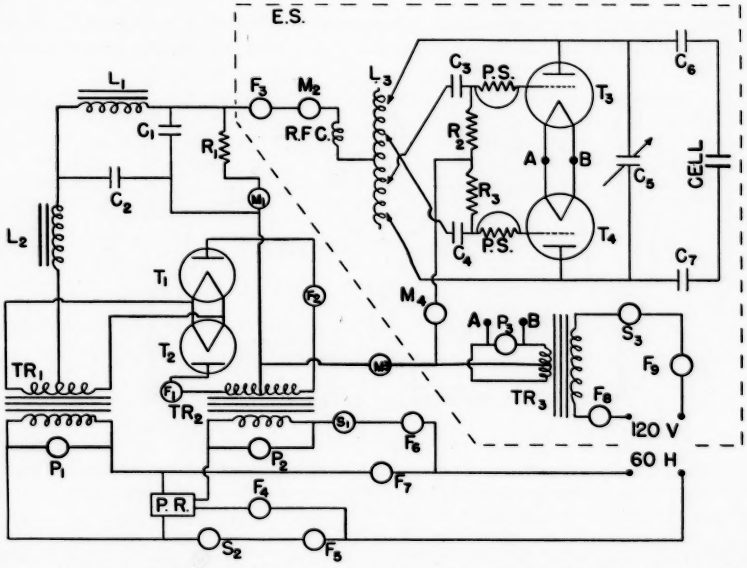

FIG. 1.—Schematic diagram of oscillator and rectifier. *E.S.* = electrostatic shield surrounding oscillator, *F* = fuse, *H* = hertz (cycles per second), *M* = meter, *P* = pilot, *P.R.* = protective relay, *P.S.* = parasite suppressor, *R.F.C.* = radio frequency choke, *S* = switch. T_1, T_2 = type 866-A rectifier tubes, T_3, T_4 = type 808 triode oscillator tubes. A Variac (not shown) in the primary of TR_2 permitted adjustment of the rectified voltage from 0 to 1500.

cancel when properly balanced. The *L–C* ratio was low (except at 60 Mc), which also reduced the harmonic content.

The water jacket and associated cooling apparatus is shown in Figure 2. The temperature at the outlet (T_2) did not exceed that at the inlet (T_1, approximately 25° C.) by more than 0.5° C. in any of the experiments.

The heterogeneous electric field was obtained by substituting for the two circular plates of the cell (Fig. 2) a lead zone-of-one base (diameter of base = 35 mm.) and a tapered rod terminating in a sharp point at the zone's center of curvature (r = 24 mm.). Two capillaries at a time were suspended perpendicular to the field axis, 10 mm. from the pointed electrode, and exposed for 40 minutes. The capillaries were prepared with

especially small quantities of medium in order that the swimming movements of the inclosed organisms would be limited to the central part of the field.

Another set of two to four capillaries was placed within a 2-inch length of ordinary 4-mm. glass tubing, and the whole was suspended in the center of the air-core inductance (L_3, Fig. 1) by means of a silk thread. This method of utilizing the flux within the tank coil provided a magnetic field essentially like that set up in the inductothermic apparatus described by Merriman, Holmquest, and Osborne (1934) and by Kling (1936), where an extrinsic applicator coil was used instead of the conventional condenser plates. For our purpose it was of advantage to employ both electric and magnetic fields at the same time on different organisms.

Between 12 and 27 Mc (25–11 m.), we measured frequency with a G.R. Model 358 wavemeter and in the neighborhood of 60 Mc (5 m.), with a G.R. Model 458 wavemeter.

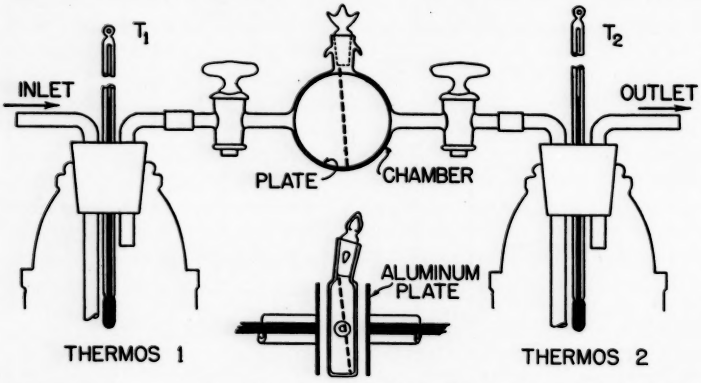

FIG. 2.—Diagram of water jacket and connections. The position of the capillary is indicated by the broken line. The lower center figure shows the relative positions of the condenser plates, water jacket, and capillary. The colpidia were confined to the mid-portion of the capillaries.

Neither of these responded in the intermediate range. To set the oscillator at 30 Mc (10 m.), we constructed an absorption wavemeter consisting of a small coil in series with a midget condenser and a 1/25-watt neon bulb mounted on Micalex and calibrated it against a G.R. Model 605-A standard-signal generator. This was done in the Marcellus Hartley Laboratory of Columbia University.

FIELD STRENGTH AT THE ORGANISM

There is considerable confusion in the literature of radiotherapy on the specification of dosage. This was initiated when early designers rated their machines in watts. The difficulty with the watt-minute as a unit of dosage is that it is impossible to know the wattage output of an oscillator without involved measurement. This cannot be done by the manufacturer and supplied as a part of the calibration of the machine because the output coupling differs with every movement of the wires or plates and with the treated material. Furthermore, an appreciable fraction of the power is often in harmonics and parasites, particularly in machines using raw alternating current on the anodes. We have used a different measure of the field at the organism—the volt per centimeter.

This is the practical unit of electric field strength or force on a unit charge. A satisfactory unit of dosage is then the volt-minute per centimeter.

The corresponding magnetic unit is the gauss-minute. The space average value of the magnetic field strength within a magnetized medium is equal to the quantity symbolized by $\boldsymbol{B}$ and called the "magnetic flux density" (Frisch, von Halban, and Koch, 1938).

It is the purpose of this section to evaluate in practical terms the strength of the field at the organism in each of the experiments. The symbols employed in the derivations are defined as follows:

A, C, P, Q	constants	l	*see* Figs. 3 and 4
a, b	*see* Fig. 3	N_n	Neumann function, order n
$\boldsymbol{B}$	flux density, gauss	q	charge, esu
c	$3 \cdot 10^{10}$ cm. per second	r	radius, centimeters
$\boldsymbol{E}$	electric field strength, esu	s	perpendicular (senkrecht)
e	empty (subscript)	u	velocity, centimeters per second
$\boldsymbol{F}$	force, dynes	V	potential, esu
f	frequency, hertz (cps)	x, y	Cartesian co-ordinates
I	current, amperes	α	$K\omega^2 - 4\pi\gamma j\omega$
J_n	Bessel function, order n	γ	conductivity, esu
j	$\sqrt{-1}$	μ	permeability
K	dielectric coefficient	ϕ	*see* Fig. 3
		ω	periodicity ($2\pi f$)

The total force on a single charge in both electric and magnetic fields is

$$\boldsymbol{F} = q\left(\boldsymbol{E} + \frac{1}{c}\,\boldsymbol{u} \times \boldsymbol{B}\right). \tag{1}$$

The quantities $\boldsymbol{E}$ and $\boldsymbol{B}$ of equation (1) are to be evaluated for the particular case of a capillary within a high-frequency field.

a) Electric field inside air-cooled capillary.—In 1864 Maxwell showed that most, if not all, macroscopic problems in electricity are determined by applying the proper boundary conditions to the general solution of four vector-differential equations. Assuming all media in the field (Fig. 3) to be homogeneous and isotropic, we obtain from Maxwell's equations:

$$\nabla^2\boldsymbol{E} + \frac{\mu}{c^2}\,(K\omega^2 - 4\pi\gamma j\omega)\boldsymbol{E} - \nabla\nabla \cdot \boldsymbol{E} = 0\,. \tag{2}$$

The last term—namely, the gradient of the divergence of $\boldsymbol{E}$—we set equal to zero, since it is assumed that no free charge is present anywhere in the field. The general solution is

$$E_{x1} = \sum_{n=0}^{\infty} (A_{nx1}\cos n\phi + C_{nx1}\sin n\phi)[P_{nx1}J_n(\alpha, r) + Q_{nx1}N_n(\alpha, r)]\,, \tag{3}$$

with similar expressions for E_{y1}, E_{x2}, E_{y2}, E_{x3}, and E_{y3}.

While considerations of symmetry simplify the application of the boundary conditions, the exact solution is very involved. Fortunately, approximate methods lead to a

solution sufficiently exact for our purposes. The contribution of the imaginary term in equation (2) is negligible, and the relaxation period of any region is very small when

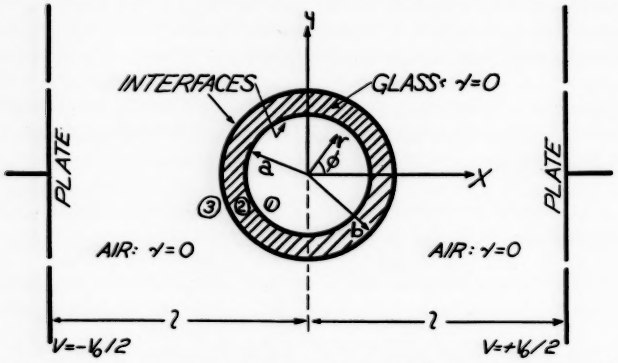

FIG. 3.—Schematized cross-section of an air-cooled capillary within the electric field

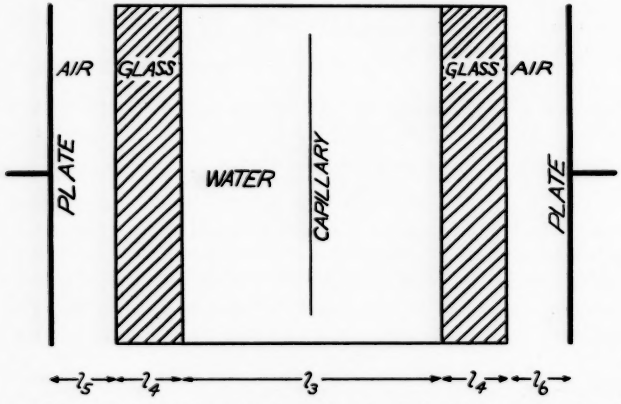

FIG. 4.—Schematized vertical section through water jacket with capillary in position

compared with the period of the oscillator. Therefore, we simplify equation (2) to the form:

$$\nabla^2 V = 0 . \tag{4}$$

The boundary conditions are: (1) $V_1 = 0$ at $r = 0$, (2) $V_3 = -E_e r \cos \phi$ at large r, (3) $V_1 = V_2$ and $K_1 E_{s1} = K_2 E_{s2}$ at $r = a$, and (4) $V_2 = V_3$ and $K_2 E_{s2} = K_3 E_{s3}$ at $r = b$. The exact solution of equation (4) shows that the field within the capillary is uniform, in the x-direction (i.e., $E_{y1} = 0$), and, when $l \gg a$, is equal to

$$E_{x1} = \frac{4K_2K_3E_e}{\frac{a^2}{b^2}(K_1 - K_2)(K_2 - K_3) + (K_1 + K_2)(K_2 + K_3)} . \tag{5}$$

TABLE 1

COMPARISON OF MEAN DAILY DIVISION-RATES IN TREATED AND CONTROL SERIES*

Frequency / Wave Length	13.0 Mc (23.1 m.)	19.1 Mc (15.7 m.)	30.0 Mc (10.0 m.)	57.2–58.8 Mc (5.1–5.25 m.)
Stock series	6.8±0.21			
Control	6.2±0.23			
Difference	0.6±0.31			
Percentage difference	9.7			
R series	6.4±0.24	7.0±0.23	8.0±0.16	6.2±0.13
Control	6.2±0.23	7.5±0.30	7.9±0.16	6.7±0.12
Difference	0.2±0.33	−0.5±0.38	0.1±0.23	−0.5±0.18
Percentage difference	3.2	−6.6	1.3	−7.5
Field intensity	12–13	14–16	9.3–11	10–11
WR series	5.8±0.30†	7.2±0.23	7.9±0.20	6.1±0.17
Control	6.4±0.91†	7.5±0.28	8.0±0.16	6.7±0.12
Difference	−0.6±0.96	−0.3±0.36	−0.1±0.26	−0.6±0.22
Percentage difference	−9.4	−4.0	−1.3	−8.9
Field intensity	28		32	31
WR-A series		6.8±0.25	7.8±0.17	6.5±0.12
Control		7.4±0.27	8.1±0.09	6.7±0.12
Difference		−0.6±0.37	−0.3±0.19	−0.2±0.17
Percentage difference		−8.1	−3.7	−3.0
Field intensity		36	36	29
WR-B series		6.8±0.26	7.4±0.13	6.5±0.19
Control		7.8±0.16	8.1±0.09	6.7±0.12
Difference		−1.0±0.31	−0.7±0.16	−0.2±0.24
Percentage difference		−12.8	−8.6	−3.0
Field intensity		35	31	30
WR-C series		6.7±0.22	7.8±0.19	6.7±0.13
Control		7.7±0.18	8.0±0.13	6.9±0.13
Difference		−1.0±0.28	−0.2±0.23	−0.2±0.18
Percentage difference		−13.0	−2.5	−3.0
Field intensity		41	30	30

* The negative differences indicate that the division-rates in exposed series were less than the controls. The field intensity varied for each of the two or three water-cooled series (*R*) in each group; only the extremes are listed. Field intensity in peak volts per centimeter.

† Mean determined for 6 days only (see Fig. 5).

b) *Electric field inside water-cooled capillary.*—Neglecting edge-effects, the field in region n of the homogeneous, isotropic system of Figure 4 is uniform and equal to

$$E_n = \frac{V}{\sum_{i=1}^{n} l_i \frac{K_n}{K_i}}. \qquad (6)$$

Combining equations (5) and (6), the field within a capillary placed in a uniform water jacket is

$$E_{x1} = \frac{4K_2K_3V}{\left[\frac{a^2}{b^2}(K_1 - K_2)(K_2 - K_3) + (K_1 + K_2)(K_2 + K_3)\right]\left[l_3 + 2l_4\frac{K_3}{K_4} + 2l_5\frac{K_3}{K_5}\right]}, \quad (7)$$

where l_5 is taken equal to l_6.

c) Magnetic field inside air-cooled capillary.—The magnetic field strength between two plates connected to an oscillator is not appreciable below a frequency of about 3,000

TABLE 2

THE TOTAL NUMBER OF GENERATIONS PRODUCED IN 48 HOURS BY COLPIDIA EXPOSED TO MAGNETIC AND INHOMOGENEOUS ELECTRIC FIELDS*

		Frequency			
		15 Mc	20 Mc	30 Mc	60 Mc
Control	First series	10.8	16.2	16.0	14.2
	Second series	9.6	14.8	15.8	15.2
	Mean	10.2	15.5	15.9	14.7
Inhomogeneous electric field	First series	9.6	16.0	14.6	18.6
	Second series	10.2	14.0	16.0	17.0
	Mean	9.9	15.0	15.3	17.8
Homogeneous magnetic field	First series	10.4	16.2	16.6	16.4
	Second series	10.4	15.8	14.8	15.0
	Mean	10.4	16.0	15.7	15.7
	Field intensity	5.7 gauss	3.2 gauss	1.9 gauss	.....

* Magnetic field intensity in peak gauss.

Mc (Mason and Weaver, 1929). The highest frequency in these experiments was 60 Mc. At the center of a coil, the field strength is approximately

$$B = \tfrac{4}{10}\pi\mu NI, \quad (8)$$

where here N means the number of turns per centimeter.

d) Inhomogeneous electric field.—In order to make dE/dx as large as possible, we made the electrode point very sharp, thereby sacrificing our ability to calculate it.

e) Numerical values.—The dielectric coefficient of pyrex glass is 4.8. Goldsmith (1937) and Knerr (1937) have shown that the index of refraction of water (n) is still about 9, even at a wave length of 24 cm. Therefore we take the dielectric coefficient of water (K) as 81, the same as its direct-current value, since $n^2 = \mu K$. The permeability (μ) of water and glass is 1. The dielectric coefficient of dilute electrolytes has been measured at high frequencies (Holzer and Weissenberg, 1935) and found to be the same as water.

No anomalous dispersion is to be expected at the frequencies employed. The peak radio-frequency voltage across the tank of an unloaded push-pull oscillator is approximately twice the value of the applied direct-current voltage. We measured the circulating current in the tank coil (except at 60 Mc) with a radio-frequency ammeter. By putting these data and the physical dimensions into equations (5), (7), and (8), we obtain the values listed in Tables 1 and 2.

A typical capillary had an outside diameter of 150 μ, a wall of 27 μ, and was filled with medium for 15 mm. of its length.

RESULTS

a) Homogeneous electric field.—The culture records from the homogeneous electric field experiments are summarized graphically in Figures 5–8, inclusive. The ordinates represent divisions per day, while the abscissas indicate successive days. Each curve

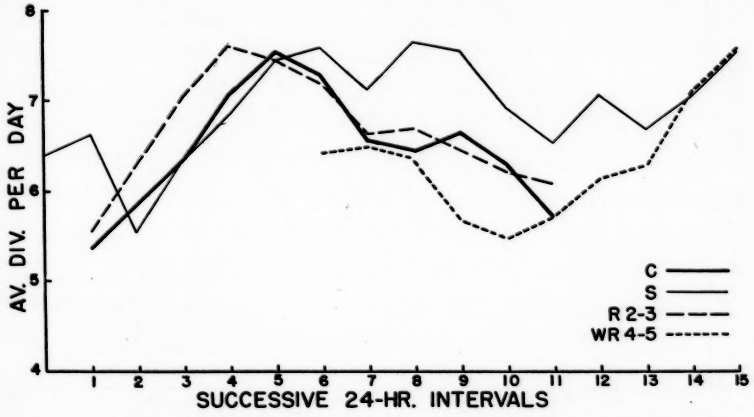

FIG. 5.—Culture records of colpidia exposed to 13.0 Mc homogeneous electric fields. Curves *C* and *S* represent one series each of control and stock cultures. Two series each of water-cooled (*R* 2–3) and air-cooled (*WR* 4–5) exposures are shown.

represents a 3-day moving average of the number of divisions in 24 hours for one or more sister series, i.e., series established from sister organisms treated under identical conditions. To illustrate: the curve *WR* 15–16–17 (Fig. 6) is the record of three air-cooled series; each point on the curve therefore gives the mean number of divisions for fifteen individual cultures taken 3 days at a time. It should be noticed that only a magnified portion of the ordinate axis is shown in each figure.

The division-rates of the "stock" series (*S*), one control series (*C*), and the experimental series treated at 13.0 Mc are compared for a period of 11 days in Figure 5. The organisms comprising the control and water-cooled series (*R* 2–3) were taken from the same stock culture. The air-cooled series (*WR* 4–5) were derived from the control strain on the sixth day.

The division-rates of organisms exposed at a frequency of 19.1 Mc are illustrated in Figure 6. One control series (*C*) and the water-cooled series (*R* 12–13–14) were estab-

lished from the stock series whose record is not included in the figure. At the end of the first 24-hour period, a group of colpidia were isolated and treated without the water bath. Three sister series (*WR* 15–16–17) were established from this group.

Exposures were also made to determine whether there are cumulative effects of successive treatments. At the end of the first 24 hours, colpidia from series *WR* 15 were given a 40-minute air-cooled exposure. Curve *WR* 15-*A* is the record of one series set up in this manner. Similarly, *WR* 15-*B* was derived from *WR* 15-*A*, and *WR* 15-*C* from *WR* 15-*B* at successive 24-hour intervals. Thus, on the sixth day of the experiment shown in Figure 6, there were ten series, a total of fifty cultures. This number of cultures represents but one-third of the organisms actually exposed at this frequency.

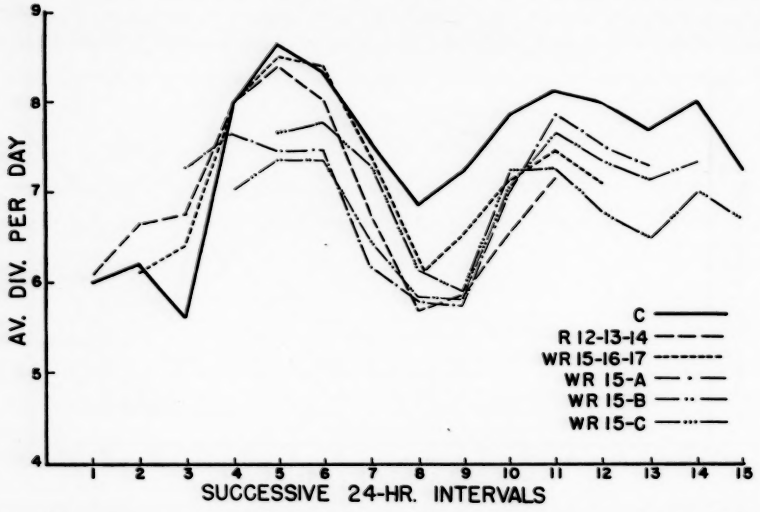

FIG. 6.—Culture records of colpidia exposed to 19.1 Mc homogeneous electric fields. One control series (*C*) and three series each from water-cooled (*R* 12–13–14) and air-cooled (*WR* 15–16–17) exposures are plotted. Curves *WR* 15–*A*, *B*, and *C* are single series of successively exposed organisms.

Twenty-two other exposed individuals were carried only to the forty-eighth hour. Their 48-hour records agree favorably with those shown in the figure.

Figures 7 and 8 are culture records of individuals exposed at 30.0 and 57.2 Mc, respectively. In order to attain the latter frequency, it was necessary to eliminate the tuning condenser (C_5, Fig. 1) and to make all adjustments by shifting the taps on the tank coil (L_3, Fig. 1). In view of the task of balancing the oscillator in this manner, it was not retuned after the removal of the water jacket. Accordingly, the water-cooled series in Figure 8 were exposed at 57.2 Mc, and the air-cooled series at 58.8 Mc.

The superimposed companion curves in each figure reveal a decided tendency to shift in comparable directions from day to day. In the six curves of Figure 6, for example, there are two maxima—one on the fifth and a second on the eleventh day. It is our belief that this conformity indicates uniform environmental conditions on a given day

and, further, that the differences in division-rate from day to day are due to variations in the bacterial content of the 24-hour medium. It was hardly feasible to attempt bacterial control in conjunction with the isolation-culture method. However, as long as the haphazard method of seeding the freshly made medium provided a favorable environment, its precise bacterial content was of no great consequence, since all cultures running on the same day were made up from the same batch of medium.

Interestingly enough, it is apparent that, with few exceptions (e.g., *WR* 25-*B*, Fig. 7), the curves of the water-cooled and air-cooled series are closely related and do not vary consistently in one direction from each other. For reasons to be considered in a later section, the temperature rise in the air-cooled capillaries was not sufficient to impair the subsequent reproductive capacities of the inclosed organisms.

More to the point, however, is the reproductive behavior of the exposed organisms in relation to the controls. If due regard is given to the magnified ordinates in the line

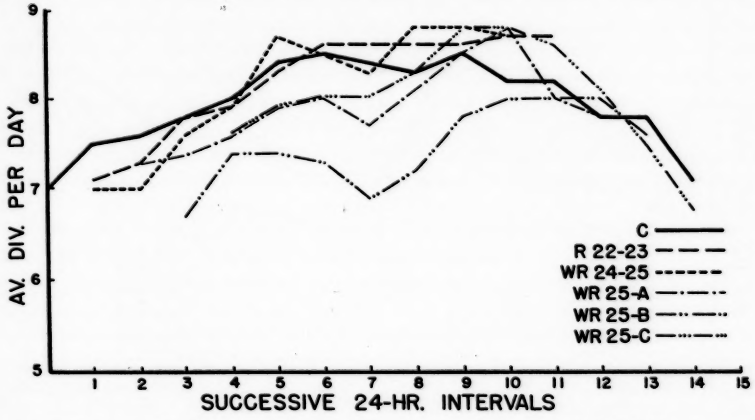

FIG. 7.—Culture records of colpidia treated with 30.0 Mc homogeneous electric fields. Curve *C* represents two control series.

graphs, the differences between the curves of experimental animals and the controls for corresponding periods are comparatively small. Although the magnitude of the differences may be of no consequence, the consistently diminished fission-rate in most of the exposed series introduces an element of doubt which must be taken into consideration.

An objective evaluation of these differences may be obtained from Table 1. Each value represents the mean number of divisions per day, together with its probable error, for 13-day culture records of from one to three series. The computations were made directly from the protocols, without regard to moving averages. The values of the controls are given for corresponding periods. Of the seventeen exposed groups, the mean rate of division exceeds the controls in two cases. In seven cases it is 0–5 per cent less than the controls, six are 5–10 per cent below, and two are 12–13 per cent below the controls. On purely statistical grounds we are unable to urge that the differences are significant. The differences between the values for treated and control organisms in Table 1 are three times greater than the probable error of the differences in only three of the seven-

teen cases. However, the confidence with which we regard the differences as of no consequence rests upon supplementary evidence.

The differences are not proportional to the length of exposure in the successively treated cultures. This is illustrated in Figures 7–8, where the *WR-C* series show an almost consistently higher division-rate than their two immediate predecessors (*WR-A*, *WR-B*).

Furthermore, three colpidia were taken from the 24-hour *WR-C* series in each experiment, sealed into capillaries, and exposed continuously for an additional 6 hours. For 48 hours afterward, in isolation cultures, they appeared to be as vigorous as the parent strains. Perhaps even more indicative is the fact that they averaged approximately two divisions each while under the influence of the high-frequency field. As a further test of increased dosage, the strength of the field at 30.0 Mc was increased by a factor of 5 (to approximately 150 volts per centimeter). This was done by reducing the distance between

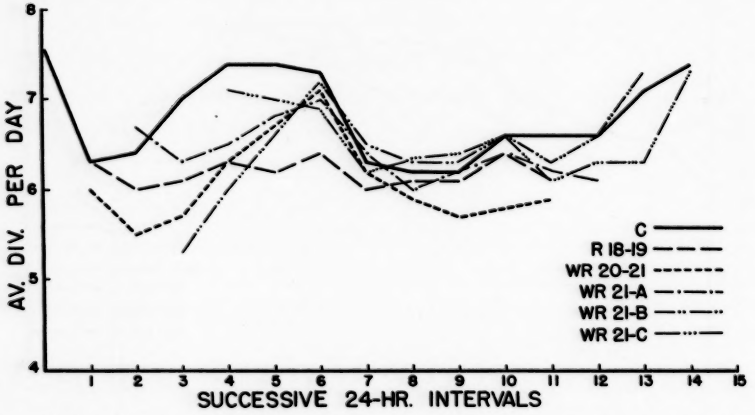

FIG. 8.—Culture records of colpidia exposed to 57.2–58.8 Mc homogeneous electric fields. Two series constitute the control curve (*C*).

the plates from 20 mm. to 4 mm. Three capillaries made up from *WR* 25-*C* on the twenty-fourth hour were suspended between the plates and given a 40-minute exposure. The subsequent 48 hours in isolation culture revealed nothing of interest.

The method of starting cultures from incapsulated colpidia presented a difficulty which was not completely overcome in this first group of experiments. The bacterial accumulations in isolation cultures are periodically sampled and passed on to new cultures at each transfer. The customary transfer pipette carries over to the new culture enough inoculum from the mother culture to preserve the sequence of bacteria. But, according to our procedure for preparing capillaries, the sealed-in droplet contained mother-culture fluid and fresh medium in the ratio of approximately 1 to 20. Moreover, the total volume of the dilute mixture actually seeded into the new drop culture by means of a capillary fragment was very small in comparison with that which is normally passed on in the pipette transfer.

Evidently this dilution factor was of some significance in depressing the division-rate

of many of the series, controls included, for the first few 24-hour intervals (Figs. 5–8, inclusive). Well-established isolation cultures of *Colpidium* were generally cloudy. The organisms were rounded and darkly colored. On the other hand, the cultures freshly seeded from capillary fragments were usually clear, and the colpidia slender and almost colorless for the first three or four transfers.

No attempt was made to compensate for this dilution factor when single treatments were given, for both control and exposed cultures were inoculated in the same manner. Whether or not the procedure varied sufficiently to account for the fact that single exposure series (*R* and *WR*) were preponderantly lower than the controls is conjectural. It is certain, however, that cultures established from the successively treated colpidia were affected. Each time, the new culture received a more dilute sample of mother-culture fluid taken from cultures established only 24 hours earlier and transferred at the first 12-hour interval. The condition was offset to some extent by fortifying each new culture with a pipette drop taken directly from the parent culture. In later experiments (Table 2), all the cultures were started with a supplementary drop from some other well-established culture. The results were more gratifying than before, in that the division-rate in exposed series was not predominantly lower than that of the controls.

b) Heterogeneous electric and homogeneous magnetic fields.—In order to explore further the possibility of affecting *Colpidium* within the selected range of frequencies, a limited number of organisms were exposed to inhomogeneous electric and homogeneous magnetic fields. The control and experimental animals were propagated in isolation cultures for only 48 hours after liberation from the capillaries. Beyond this point they were allowed to develop as mass cultures. Because of the few experiments which we were able to make, the results are indicative rather than conclusive.

Table 2 gives the total number of generations produced in 48 hours by individual series, each the mean of five lines. For the most part, the number of generations produced in 2 days by those treated at each frequency are in close agreement with the controls. Those exposed in the inhomogeneous electric field at 60 Mc, however, show a suspiciously increased division-rate. In the absence of further data from isolation cultures, it is necessary to rely upon records from the mass cultures for additional information. These records indicate that the observed differences at the forty-eighth hour are probably of little significance.

DISCUSSION

There appear to be two modes of energy transfer by which a high-frequency field may affect tissues or organisms. The first is that of heating the protoplasm, the medium, or both in the manner described by McClennan and Burton (1930). According to this method, different biological structures may be affected differentially, depending upon three factors: frequency, dielectric coefficient, and conductivity.

The energy content of an organism may also be altered—not by heating in the foregoing sense, but through an electrical mechanism which depends upon the presence in the organism of free charges or chemicals with dipole moments. Given either or both of these conditions, it is conceivable that the forced vibrations may lead to observable changes in the internal organization. If the homogeneous field intensity is sufficiently great, a small dipole may oscillate longitudinally without translation; i.e., it may alternately elongate and contract. The dipole movements are both longitudinal and translational in an inhomogeneous field. In translational vibration the dipole, as a whole,

moves small distances on either side of its mean position. We do not believe, however, that any of the frequencies employed were high enough to cause such free oscillations or "resonance" in our material.

Although a diversity of opinion prevails in respect to the question of nonthermal effects of high-frequency fields upon living material, the most assuring evidence appears to have come from those investigations which have dealt with small organisms, individuals, or cells whose normal environment can be simulated *in vitro* and in which the temperature of the surrounding medium is immediately assumed.

Kahler, Chalkeley, and Voegtlin (1929) exposed 150–400-cc. suspensions of *Paramecium caudatum* in electric and magnetic fields at 10 Mc and in electric fields at 75 Mc for periods of 1–4 hours. Also, Gale (1935) used the electric field at 15, 30, and 50 Mc for 1–6 hours on *P. caudatum* and *Chilomonas*. These workers found that the protozoa were destroyed at 41–43° C. when treated without temperature control. Slow direct heating with a water bath produced identical results. But when the suspensions were kept at sublethal temperatures by frequent chilling or by suspending the organisms in a nonconducting, nonheating medium (Kahler *et al.*), no after-effects were detected.

Jellinek (1936) attempted to exclude large-order heating effects by working with an extremely weak field. *Paramecium* and *Vorticella*, in single-drop cultures, were immobilized or destroyed immediately at 100 Mc. In view of his results with the same field upon other organisms, our own experiences with *Colpidium* in small quantities of fluid, and those of others on *Paramecium* at somewhat lower frequencies but greater field intensities, it seems probable that Jellinek's unusual results with the protozoa were due to other causes, perhaps contaminated glassware.

Kindred work on other microörganisms emphasizes the need for an efficient method of heat control for both organism and medium in order to exclude the possibility of selective or preferential heating.

Haase and Schliephake (1931) demonstrated that *Staphylococcus* cultures maintained at 55° C. in an electric field were destroyed in from 6 to 15 minutes, according to the frequency used. Thirty minutes were required for sterilization in a water bath at the same temperature. These authors believe that some peculiar effects upon the colloidal organization of the bacteria are involved.

By alternately exposing and cooling cultures of different bacteria, Whitney and Page (1935) observed that, when the media were not heated appreciably, the bacteria continued to live. Similarly, Breitweiser (1935) subjected bacteria on chilled media to intense fields at several frequencies. These produced luxuriant growths. After exposure to moderately strong fields on pre-warmed media, only a few scattered colonies of the bacteria were obtained. The authors attribute the lethal action to thermal changes in the media. Whitney and Page refer to the effect as a "thermal sterilization of the medium." They were also able to show that, at certain frequencies, temperature increased more rapidly within frog tadpoles than in the distilled water surrounding them, whereas a tap-water medium heated as readily as the animals. For colonies of bacteria or frog tadpoles it is therefore possible to obtain effects which are not proportional to the thermal changes in the medium. The experiments of Bachem (1935) on *Bacillus prodigiosus* and *Saccaromyces cerevisiae* are further suggestive. For a series of frequencies between 20 and 60 Mc, the rate of heating in these microörganisms was less than that of the fluids in which they were suspended. The reverse obtained between 60 and 85 Mc for the yeast and apparently above 85 Mc for the bacteria.

The question now arises: What light do our experiments throw upon the existence of an electrical mode of energy transfer distinct from heating? In order to avoid excessive temperature gradients in any part of the reacting system, our object was to eliminate, as far as possible, all temperature changes within the organism or medium during the course of treatment. To this end the material was prepared in the form of minute cylinders (capillaries) in which cooling is inversely proportional to, and heating a direct function of, the radius (Gale, 1935). The use of individual colpidia, instead of mass cultures, also excludes the so-called "antenna effect," which, as a function of population density, emphasizes the importance of numbers. Whitney and Page (1935), for example, demonstrated that organisms in groups were more responsive to high-frequency fields than single individuals.

Relatively large quantities of the rice-salt medium (pH 5.1) had a high rate of heating, as shown by a series of 40-minute determinations at each frequency setting. To cite one example: at 30 Mc the mean temperature change for 13.1 cc. of medium in the closed-off water jacket was 58.8° C., much greater than that required to bring the temperature of the medium (initially at 25° C.) up to the thermal death-point of *Colpidium* (42 ± 1° C.). But at the same frequency, approximately 0.3 cu. mm. of the medium and one organism within an air-cooled capillary failed to heat appreciably, even after 6 hours of continuous treatment.

Observations on the colpidia exposed were fragmentary because direct examination of microörganisms within the field was not possible. The capillaries were examined within a few seconds after the power was turned off. In the water-cooled capillaries the behavior of the colpidia was identical with the controls in all respects. Their motor activities were normal, and their subsequent divisions were more or less synchronous with the controls. In the air-cooled capillaries slight differences were sometimes evident in the more rapid movements of the organisms and in their tendency to divide in advance of the controls. These were the only indications of temperature variations within the air-cooled capillaries.

SUMMARY

To investigate the possible existence of an effect of high-frequency fields on biological organisms, which cannot be shown to result entirely from heating, 129 colpidia were exposed to high-frequency electric and magnetic fields under the conditions of constant temperature and controlled environment. As a qualitative measure of change we used direct microscopic observations before and after exposure. For a quantitative measure we compared the mean rate of fission of the exposed organisms with the nonexposed controls otherwise treated identically. Three types of field were employed: a homogeneous electric field, a homogeneous magnetic field, and a highly inhomogeneous electric field.

From the results of these experiments we conclude that there is no large-order effect on the physiological organization of *C. campylum*, as evidenced by its subsequent division-rate, which can be attributed to a nonthermal mode of energy transfer from high-frequency electric or magnetic fields in the 13–60 Mc range of frequencies.

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NUTRITIONAL STUDIES OF PARAMECIUM MULTIMICRONUCLEATA.[1] I. QUANTITATIVE AND QUALITATIVE STANDARDIZATION OF THE FOOD ORGANISM

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CERTAIN experimental studies of allelocatalytic effects in protozoa feeding on bacteria point out a need for quantitative standardization of the food. McPherson, Smith, and Banta (1932) found that groups of *Paramecium caudatum* had a higher fission-rate than did singles when in a strong medium (high bacterial concentration) and that in a weak medium (low concentration of bacteria) the reverse was true. From this they conclude that conflicting results in the study of allelocatalysis may have arisen, in part at least, from differences in strength (food value) of culture medium used by different workers. Smith (1932), studying the strength of the culture medium as a factor in fission-rate of *P. caudatum*, also concludes that the concentration of the medium (presumably excess or deficiency of food) seems to be the predominating factor limiting the fission-rate. And Johnson (1933) in his studies on population densities in *Oxytricha fallax* has shown that many of the allelocatalytic results can be explained on a basis of the ratio existing between the infusorian and the bacterial population. It is therefore evident that some studies on the quantitative standardization of the foods of bacterial feeders are necessary in order that reproducible results may be obtained in future experimental work dealing with such organisms.

In most experimental studies on holozoic protozoa the "standard loop method" has been used for measuring quantities of food bacteria. This method, however, does not permit a rigid control of bacterial concentrations. Giese and Taylor (1935) endeavored to standardize the quantity of food for *Paramecium multimicronucleata* more accurately by comparing a mixture of the food organism, *Pseudomonas ovalis*, in their basic lettuce medium for culturing the ciliate, to a suspension of 0.025 per cent $CaCO_3$. This served as a rough control. However, in view of the fact that certain protozoa among the bacterial feeders may now be physiologically standardized in large numbers because their excystment time is known, it is clear that a rigid control of the quantity of food is also desirable for further standardization of these experimental cultures. For that reason certain mechanical devices—the suspensiometer and turbidimeter—have been tested here for their accuracy in quantitative measurement of bacterial suspensions.

THE SUSPENSIOMETER

Gates (1920) reported that the opacity of a bacterial suspension could be measured by the length of the column of the suspension required to cause the disappearance of a wire loop; and, by means of a simple formula, the measured opacity could be translated into terms of bacterial concentration per cubic centimeter and so made comparable to other suspensions of the same organism. An instrument for measuring the concentration of bacterial suspensions based on the principle of Gates is now on the market and is

[1] The writer is greatly indebted to Professor W. H. Johnson for suggesting this investigation and for his advice while carrying it out.

called a "suspensiometer." Such an instrument was tested and found inadequate for accurate quantitative measurement of suspensions of bacteria. Very milky suspensions of bacteria are required to obtain a sharp end-point for the disappearance of the image of the wire loop. The use of such dense bacterial suspensions makes it impossible to have consistent agreement between the numbers of bacteria present per cubic centimeter and the opacity of the suspension. In testing this device, all the bacterial suspensions were prepared from a single pedigreed strain of bacteria, which had been cultivated under uniform conditions for a definite period before use.

THE TURBIDIMETER

Richards and Jahn (1933) and Clifton, Mueller, and Rogers (1935) have described photoelectric turbidimeters which measured bacterial suspensions with a relatively high degree of accuracy. Fundamentally these devices are identical, as each employs a Weston Photronic photoelectric cell, the current output of which is essentially a linear function of illumination. Therefore, these cells were used to measure the light absorbed by a suspension of microörganisms.

In order to test the principle of the turbidimeter, an instrument was constructed which combined some features of each of the two described by the above-mentioned investigators. The apparatus consisted essentially of a light source, a 15.0 × 1.5-cm. culture tube for the bacterial suspension, a Weston Photronic cell, and a microammeter.

The apparatus, patterned somewhat after the "vertical type" of Richards and Jahn, was housed in a Leitz-Wetzler microscope cabinet having inside dimensions of $5\frac{7}{8} \times 6 \times 11\frac{3}{4}$ inches. The light source (a 50-candle-power, 6–8-volt Mazda automobile lamp) was mounted in a Spencer substage lamp box containing a reflector. This metal lamp box in turn was secured to the inner side of the top of the cabinet so that the lamp filament was brought directly over the mouth of the culture tube inserted into a tight-fitting hole passing through a solid wooden block $3\frac{1}{4} \times 3\frac{1}{4} \times 4\frac{1}{2}$ inches. This block was supported on a shelf having a small 1.4-cm. beveled hole directly in line with that in the block. This arrangement permitted only light from the lamp passing through the entire length of the culture tube to fall on the Photronic cell, seated in a radio-tube socket, directly beneath and touching the shelf. The beveled hole in the shelf permitted the culture tube almost to touch the partially exposed face of the Photronic cell blocked off so that scattered light in dense suspensions would not fall over its surface. The culture tube was also held in position by a secondary shelf, which, sliding into place just beneath the lamp, helped to shut out any extraneous light which might otherwise pass below. Any other scattered light was absorbed by the black walls of the closed cabinet.

The intensity of the light source was maintained by means of a slide-wire resistance in series with the lamp and storage battery supplying the current for the circuit. The battery was continually charged by means of a General Electric Tungar battery charger during the course of the experiment.

The current generated by the Photronic cell was measured by a Weston Model 600 microammeter having a range of 0–500 microamperes. The position of the slide-wire resistance was adjusted so that the "standard" culture tube, when held in a definite position (noted by markings) and containing 20 cc. of sterile artificial pond water, gave a constant reading of 300 microamperes. When the intensity of the light was so regulated, the microammeter reading gave directly the percentage of light transmitted by any 20-cc. suspension substituted for the sterile medium in the "standard" culture tube.

The instrument was calibrated with suspensions of 4-day-old cultures of *Pseudomonas fluorescens* prepared by adding 8 "standard" loops to 20 cc. of sterile balanced-salt medium. The total number of bacteria in 1 cc. of each suspension was determined by the pour-plate method. Seven or 8 pour plates were always prepared for each count from the proper dilution of the suspension.

Clifton *et al.* (1935) reported for their tubidimeter that "a linear relationship between the bacterial counts and the logarithms of the microammeter reading was observed over a wide bacterial concentration range." Their findings were verified by the use of the instrument described above, to the extent that in a very narrow range of bacterial concentrations a straight-line relationship was found when the bacterial counts were plotted directly against the microammeter readings. Such linear relations were found to hold for the 4-day-old cultures of *Ps. fluorescens* grown at 26° C. on Difco Bacto nutrient agar.

As Richards and Jahn (1933) also reported that their turbidimeter estimates of yeast cultures were more accurate than the counts made with the haemocytometer, it is evident that turbidimeters do offer a means of standardizing more rigidly the density (quantity) of some food suspensions used in experimental protozoan studies. Therefore, their use should greatly enhance further standardization in experimental studies using protozoa having an established excystment time, as both the age of the protozoa and the quantity of a specific particulate food could be controlled.

As has already been stated, in most experimental studies on protozoa the standard loop method has been used to control the quantity of bacterial food required. In these studies it has been assumed that, even though there are slight variations in food quantity resulting from the use of the loop method, such variations do not influence the experimental culture, because some of these experimental animals seem to tolerate bacterial crowding within certain limits without effect upon their reproductive rate. Barker and Taylor (1931) in their investigations on *Colpoda* state:

> If too many bacteria surround the protozoa, the latter are adversely affected as shown by a strongly reduced division rate. . . . . In an exceedingly dilute suspension of bacteria the protozoa show reduction in size and division rate. . . . . Between these two extremes is a relatively wide range of bacterial suspension densities in which the division rate and size of *Colpoda* are maximal and approximately constant.

Johnson (1933) in his studies on *Oxytricha* showed that there is an optimum concentration of food bacteria for the maximum rate of reproduction of the experimental animal. Using the wire-loop method, he found that suspensions of *Ps. fluorescens* in densities varying from 80 to 135 millions per cubic centimeter were optimal for a given number of this hypotrich under the conditions of his experiments. Johnson (1936) investigated the course of populations of *Paramecium* in different densities of bacteria and found that during the first 24 hours of culture there was no difference in the fission-rate of either singles or groups of five paramecia in either x or $5x$ concentrations of *Bacillus subtilis* in a controlled volume of the culture fluid. It is such findings as these which indicate that certain protozoa, among the bacterial feeders, can tolerate slight differences in food concentration in daily subculture without effect upon their rate of reproduction, and which justify the use of the wire-loop method for controlling the food quantities in experimental studies on these forms.

In order to determine more conclusively the advisability of the wire-loop method, it

was decided to ascertain if bacterial crowding over a long period has an effect upon the rate of reproduction in *Paramecium*. It was felt that if in x or $4x$ bacterial concentrations, representing densities of 2 and 8 standard 1-mm. loops, respectively, in 5 cc. of an inorganic culture medium, there were no differences in the average daily fission-rates of twenty paramecia in 1-cc. portions of these food suspensions, the standard loop method would be an adequate means of quantitatively standardizing the food supply of this organism in daily subculture. The slight variations in food quantity resulting in the use of this method would not equal the wide differences in the concentrations selected here.

MATERIALS AND METHODS

In order to establish reproducible conditions in an experimental study on the effect of different food concentrations on the fission-rate of *Paramecium*, it is necessary to use (1) a nonnutrient protozoan culture medium, (2) a pedigreed strain of bacteria as food qualitatively standardized, and (3) controlled optima for all physical environmental factors. The animals used in the present investigation were taken from a mass culture of *P. multimicronucleata* which Professor W. H. Johnson has had growing in his laboratory at Stanford University for many months at room temperature in an inorganic culture medium on *Ps. fluorescens*.

The non-nutrient culture medium used was comparable to that from which the animals were taken. This balanced physiological salt medium, first reported by Osterhout (1906), has been used successfully for the culture of various ciliates. It was employed by Barker and Taylor (1931) for the culture of *Colpoda cucullus* on *Ps. fluorescens;* by Johnson (1933) for cultivating *O. fallax* on *B. subtilis* and on *Ps. fluorescens;* by Gause (1934) for growing *P. caudatum* and *Paramecium aurelia* on *Bacillus pyocyaneus;* and later by Johnson (1936) for culturing *P. caudatum* on six strains of identified bacteria. The desirability of this non-nutrient medium is twofold: (1) it is reproducible; and (2) it will not support the growth of the bacterial inoculum, as shown by Johnson (1933). This non-nutrient salt solution was diluted 1:500 with triple-distilled water, so that its salt concentration was comparable to that of pond water, or 0.012 total salt. The formula is as follows:

H_2O	1,000 gm.	$MgSO_4$	2.0 gm.
NaCl	52.0	KCl	1.15
$MgCl_2$	4.25	$CaCl_2$	0.5

One cubic centimeter of M/20 NaH_2PO_4 was added to each 30 cc. of the diluted salt solution for buffering, and the pH was brought to 6.8–7.0 with M/20 NaOH. The adjusted solution was then filtered through a Chamberland filter, as recommended by Johnson and Hardin (1938). After filtration, 5-cc. portions of the solution, in cotton-plugged tubes, were autoclaved at 15 pounds pressure for 20 minutes. These tubes were then ready for use.

For the present experiments the food organism was a pedigreed strain of *Ps. fluorescens* (U6) obtained from the bacteriology department of Stanford University. This food was qualitatively standardized throughout the experiments by cultivating the bacteria for a definite time before use at 26° C. on slants of the commercial Difco Bacto nutrient agar. This agar was selected in order to facilitate the establishment of reproducible conditions. To have the food organism a definite age (i.e., qualitatively standardized)

at the time of daily feeding of the protozoa, new subcultures of the bacteria had to be prepared daily and then incubated for the required period. The purity of these subcultures was regularly checked. At the time of feeding the paramecia, the aged bacteria were removed from the slants with a platinum loop 1 mm. in diameter and suspended in 5 cc. of the sterile salt solution. A level loopful was transferred each time, while the number of loops varied according to the experiment.

The ratio of the volume of the culture medium to the number of paramecia at the time of subculture was constant at 1 cc. to twenty animals, a ratio equivalent to one animal per drop from many pipettes. Sterile volumetric pipettes were used to carry 1 cc. of the bacterized culture medium into clean ground-glass Columbia culture dishes of 1 cc. capacity in which the ciliates were grown as isolation cultures.

The paramecia were subcultured daily by isolating twenty, with a sterile mouth pipette, from a similar culture started 24 hours earlier and grown in a moist chamber in an incubator maintained at 26°–27° C. by a thermostat. Counts of the remaining animals in each 1-day-old subculture were made under a dissecting microscope, with counting facilitated by the use of a mechanical hand counter. From the total number of progeny produced, the daily fission-rate was determined.

EXPERIMENTAL DATA

In these studies the food organism, *Ps. fluorescens*, was qualitatively standardized by being incubated either 4 or 30 days before use. And quantitative standardization, as stated above, required the use of an x, or 2-loop, concentration and a $4x$, or 8-loop, concentration of each qualitatively standardized food. With the animal-medium ratio established at twenty animals per 1 cc. of each density of the conditioned food, several animal lines were prepared for study in a given experiment. The experiments were as follows:

5 lines of *Paramecium* in x density of 4-day-old food
3 lines of *Paramecium* in $4x$ density of 4-day-old food
3 lines of *Paramecium* in x density of 30-day-old food
3 lines of *Paramecium* in $4x$ density of 30-day-old food
5 lines of *Paramecium* in $2x$ density of 4-day-old food

Five lines of Paramecium in x *density of 4-day-old food.*—During the 4 weeks these five lines of *Paramecium* were carried in this 2-loop concentration of the 4-day-old food, their composite daily fission-rate averaged 1.2. Although there was some daily deviation from this average, the line cultures, for the most part, reproduced uniformly in relation to one another; and the protozoans in each were normal in appearance, in comparison with those in a mass stock culture. The average number of progeny per

TABLE 1

FIVE LINES OF 20 *Paramecium* IN 2-LOOP DENSITY OF 4-DAY-OLD FOOD

48	46	43	34	62	92	108	52	31	27	34	53	74	58	57	60
1.2	1.1	1.0	0.7	1.5	2.1	2.3	1.3	0.5	0.3	0.7	1.3	1.8	1.4	1.4	1.5
52	60	72	55	33	37	47	47	40	51	51	40	43	46	Av.	*51*
1.3	1.5	1.8	1.3	0.6	0.8	1.1	1.1	1.0	1.2	1.2	1.0	1.0	1.1	Av.	*1.2*

day of the five lines is given in Table 1, as is the average daily fission-rate. The viable bacterial concentration of a 2-loop, or x, density of 4-day-old food in 5 cc. of the culture medium averaged 74 million bacteria per cubic centimeter, as determined by the pour-

plate method, using ten test suspensions. The concentrations of the living organisms in these test suspensions were found to be 82, 76, 87, 81, 65, 80, 54, 74, 68, and 77 millions per cubic centimeter.

Three lines of Paramecium in 4x concentration of 4-day-old food.—After a period of 6 days, during which time one of these three lines had to be renewed, all the cultures began to flourish, as did those in the foregoing *x* concentration of the similarly aged food. And, except for the introductory period, these three lines maintained a relatively constant division-rate in 4*x*, or 8-loop, concentration. Over the 4 weeks that these lines were maintained, the composite daily reproduction-rate of the protozoans averaged 1.2 fissions. Since this rate is directly comparable to that of the foregoing five-line series in the *x* concentration of this same food, this would indicate that bacterial crowding within the limits of concentration of 2 to 8 standard 1-mm. loops has little or no effect upon *Paramecium* grown in isolation cultures as carried on in these experiments. In terms of viable bacteria, a 4*x* increase in concentration is equivalent to 296 million bacteria per cubic centimeter, judging from the average number of living organisms in an *x* concentration of this food. The history of the three line cultures of *Paramecium* in the 4*x* concentration of 4-day-old food is summarized in Table 2.

TABLE 2

THREE LINES OF 20 *Paramecium* IN 8-LOOP DENSITY OF 4-DAY-OLD FOOD

33	25	11	6	12	28	65	57	38	55	49	59	84	94	100	66
0.6	0.2	0.0	0.0	0.0	0.4	1.6	1.4	0.9	1.3	1.2	1.4	2.0	2.1	2.2	1.6
72	43	54	56	32	42	47	39	49	52	44	58	65	Av.	*49*	
1.8	1.0	1.3	1.4	0.6	1.0	1.1	0.9	1.2	1.3	1.1	1.4	1.6	Av.	*1.2*	

Three Paramecium lines in x *density of 30-day-old food.*—An *x* concentration of food of this age was found to contain, on an average, 12 million viable *Pseudomonas* per cubic centimeter of the culture medium. The three lines cultured in an *x*, or 2-loop, concentration of the food organism taken from 30-day-old slants were practically uniform in their daily division-rates. And there was little deviation from the average daily fission-rate of 1.0 divisions which the protozoans maintained during the 4 weeks they were subcultured. Table 3 summarizes these findings.

TABLE 3

THREE LINES OF 20 *Paramecium* IN 2-LOOP DENSITY OF 30-DAY-OLD FOOD

42	35	40	49	34	48	48	37	71	54	49	53	39	34	33	34
1.0	0.7	1.0	1.2	0.7	1.2	1.2	0.8	1.7	1.3	1.2	1.3	0.9	0.7	0.6	0.7
26	35	42	34	43	40	38	42	37	44	53	39	40	37	Av.	*41*
0.3	0.7	1.0	0.7	1.0	1.0	0.9	1.0	0.8	1.1	1.3	0.9	1.0	0.8	Av.	*1.0*

Three Paramecium lines in 4x concentration of 30-day-old food.—The paramecia in these three lines in standardized food suspensions of 30-day-old food in 4*x* concentration had an average division-rate similar to all the experimental lines so far considered. Judging from the experiment above, this concentration would contain 48 million living bacteria per cubic centimeter. During the 30-day period in which these protozoans were cultured in an 8-loop concentration of the food organism, the average daily fission-rate was 1.0. This average rate is identical with that of the three lines in the lesser concentration of the same food so qualitatively standardized. However, in the concentrated food suspension a greater deviation from the mean was noted. It was assumed

that this deviation was due to some other factor than concentration, since the average division-rate of the organisms in both the 2- and the 8-loop densities was comparable. The results are shown in Table 4.

TABLE 4

THREE LINES OF 20 *Paramecium* IN 8-LOOP DENSITY OF 30-DAY-OLD FOOD

68	60	63	62	45	64	45	51	42	34	35	32	33	36	36	33
1.7	1.5	1.5	1.5	1.1	1.6	1.1	1.2	1.0	0.7	0.7	0.6	0.6	0.8	0.8	0.6
38	40	51	36	34	32	25	25	24	25	31	35	37	33	Av.	*40*
0.9	1.0	1.2	0.8	0.7	0.6	0.2	0.2	0.2	0.2	0.5	0.7	0.8	0.6	Av.	*1.0*

Five Paramecium lines in 2x concentration of 4-day-old food.—In order to check the experiments so far considered, another experiment was carried on simultaneously in which the concentration of food was 4 loops and the food organism was 4 days old. Over a period of 3 weeks, in which five lines were subcultured, the composite average daily fission-rate of the paramecia was 1.1. As in the other experimental series herein described, the daily fission-rate of these particular lines was relatively uniform, with only slight deviation from the average mean, as seen in Table 5. The rate of 1.1 for the check experiment, being comparable to all the other lines carried in this investigation, permits the conclusion that bacterial crowding between 2 and 8 standard 1-mm. loops has no effect upon the division-rate of *P. multimicronucleata* when grown in isolation cultures under the reproducible conditions used in the present study. However, in other concentrations or conditions it may be that bacterial crowding has an effect upon *Paramecium*, as Johnson (1933) noted for another ciliate, *Oxytricha*, using *Ps. fluorescens* as food. The results of this control experiment are shown in Table 5.

TABLE 5

FIVE LINES OF 20 *Paramecium* IN 4-LOOP DENSITY OF 4-DAY-OLD FOOD

51	44	42	44	36	42	55	55	43	47	43
1.2	1.1	1.0	1.1	0.8	1.0	1.3	1.3	1.0	1.1	1.0
37	47	60	53	56	45	44	39	42	Av.	*46*
0.8	1.1	1.5	1.3	1.4	1.1	1.1	0.9	1.0	Av.	*1.1*

CONCLUSIONS

Agreement in experimental results in studies of protozoa which feed on bacteria is difficult to obtain if standardized conditions are not employed. The use of qualitatively and quantitatively regulated foods in such investigations, along with certain protistological techniques, should lead to reproducible results. Qualitative standardization, which implies that the food organism is at a given age at the time of being utilized, may be accomplished by certain bacteriological methods. The present investigation on quantitative standardization of the food supply of bacterial feeders shows that the quantity of food organisms may be regulated by the turbidimeter described. Its use, along with qualitatively standardized food, should facilitate further regulation of the experimental cultures in those investigations using protozoa capable of being physiologically standardized because their excystment time is known.

This investigation has also shown, for a specific case in which the experimental organism cannot be physiologically standardized in large numbers, that a rigid control of the quantity of food used is not necessary to obtain reproducible results, provided other

factors are controlled. *Paramecium multimicronucleata* grown under controlled environmental conditions on qualitatively standardized food was found to maintain a more or less uniform division-rate over an extended period even though the quantity of food varied considerably within a range which may be considered optimum under the conditions described. This was found to be the case when the food supply varied between 2 and 8 standard 1-mm. loops per 5 cc. of the inorganic culture medium and when the animal-culture medium ratio was twenty animals per 1 cc. at the time of daily subculture. These findings suggest that the wire-loop method is satisfactory for quantitatively measuring the bacterial foods of *P. multimicronucleata* when an optimum amount of food is employed, because the slight variation in food quantity resulting from its use would have no effect upon the division-rate of the organism. That this is true was shown by the fact that the concentration of viable bacteria may vary between 74 and 296 millions per cubic centimeter of 4-day-old-old food and between 12 and 48 millions in 30-day-old food without affecting the division-rate of *Paramecium* subcultured daily over a period of 4 weeks. It may also be concluded from the present findings that this protozoan can use *Ps. fluorescens* as food when the latter is between the ages of 4 and 30 days and is cultivated under the conditions described.

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